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Clinical and Molecular Aspects

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For the AIE patients and their parents

'Healing is a matter of time, but also sometimes a matter of opportunity.'

Hippocrates

Voor mam en pap

Paranimfen:

Marjolein Moes
Sabine Heitman-ten Berge

The research described in this thesis was conducted at the Department of Paediatric Gastroenterology, Hepatology and Nutrition of Necker-Hospital in Paris-France, INSERM Unit 793 and University Paris Descartes V in collaboration with the Department of Paediatric Gastroenterology, Hepatology and Nutrition of the University Medical Centre Groningen and the University of Groningen. The research was financially supported by unrestricted research grants from NWO Zonmw AGIKO and the Nutricia Research Foundation.

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CHAPTER 1

INTRODUCTION AND RATIONALE, AIM AND OUTLINE OF THIS THESIS

Adapted from:

1. Ruemmele FM, Moes N, de Serre NP, Rieux-Laucat F, Goulet O. Clinical and molecular aspects of autoimmune enteropathy and immune dysregulation, polyendocrinopathy autoimmune enteropathy X-linked syndrome. Curr Opin Gastroenterol. 2008 Nov;24(6):742-8.

2. Moes ND, Ruemmele FM, Rings EHHM. Autoimmune enteropathy in children. Ned Tijdschr Geneeskd. 2011;155:A3246. Dutch.

3. (Moes N, Ruemmele FM. Autoimmune enteropathies- new insights. JPGN. In preparation).

1.1 Introduction

Autoimmune enteropathy (AIE) is a rare but severe form of immune-mediated inflammatory intestinal disease causing persistent diahrroea in young children.
AIE is characterized by 'self'-directed inflammation and the presence of auto-antibodies causing intestinal mucosa destruction that is recognizable by severe villous atrophia and T-cell infiltration of the lamina propria. AIE can be part of systemic disease with involvement of other organs. Phenotypic presentation of patients with AIE can vary widely. To date it is not known whether this is due to genotype differences. Therapeutic options are limited and disease course is often fatal early in life. The underlying molecular mechanisms of the disease are still largely unknown.
The present thesis analyzes the different phenotypes of AIE and investigates molecular mechanisms involved in the pathogenesis and treatment of AIE aiming to improve diagnostic and therapeutic strategies.
This first chapter provides background information discussed in six sub-sections: the clinical description of AIE (1.1.1), the occurrence of AIE (1.1.2) the pathophysiology of AIE (1.1.3), the molecular background of AIE (1.1.4), diagnosis of AIE (endoscopy, histology, immunology and genetics) (1.1.5) and treatment of AIE (1.1.6). In addition, the last three sections describe the rationale (1.2), aim (1.3) and outline (1.4) of the thesis.

1.1.1 Clinical Description of AIE

AIE causes marked intestinal inflammation leading to mucosa destruction that may manifest in the form of clinical symptoms such as abdominal pain, intestinal bleeding and diarrhoea. The autoimmune and inflammatory response predominantly affect the small bowel resulting in severe protein-losing enteropathy. However, the colon can also be affected; leading symptoms include defecational urge, tenesmus and presence of blood and mucus in the stools. In addition, AIE can be associated with extra-intestinal auto-immune manifestations such as insulin-dependent diabetes mellitus (IDDM), hemolytic anemia or pancytopenia, cutaneous autoimmune inflammation or tubular nephropathy.
In general, symptoms of AIE can be alleviated by immunosuppressive treatment. High-dose corticosteroid therapy reduces the intestinal inflammation, diminishes diarrhoea and makes enteral feeding possible. However, steroid medication alone is often not sufficient. Therefore, T-cell mediated immunosuppressive therapy (e.g. cyclosporin A, tacrolimus or sirolimus) is needed.
A particularly severe form of autoimmune enteropathy occurs in immune dysregulation, polyendocrinopathy, autoimmune enteropathy X-linked (IPEX) syndrome.[1–3] In this situation, AIE develops during the first months of life after a normal neonatal period. Stool volumes can be up to 100 mL/kg/day resulting in a high risk of dehydration and metabolic deregu-

lation. The systemic nature of AIE is reflected in IPEX syndrome, which involves additional autoimmune/inflammatory manifestations in other organs. Based upon the results of this thesis represented in chapter three, we distinguish two different phenotypical presentations of IPEX syndrome. In IPEX syndrome type one, the enteropathy is combined with early onset IDDM, other endocrinopathies, eczema, autoimmune hematological abnormalities or renal involvement.[3–6] This occurs in 75% of patients (see the tabel 1 in the next section of this chapter). IPEX syndrome type two is characterized by severe immune-allergic manifestations in the form of dramatic eczema and severe food allergies and occurs in 25% of cases.[3,4,7] Even with a heavy immunosuppressive regime, response to therapy in IPEX syndrome is often insufficient. The only cure is hematopoietic stem cell transplantation. However, therapeutic results are variable.[8–10] Despite current improvements in therapeutic achievements, the evolution of IPEX syndrome continues to be fatal in most children of young age (< 2 years).
Other non-IPEX syndrome forms of AIE also exist with isolated gastrointestinal (GI) involvement. Most of them are less severe and often respond to immunosuppresive medication (like azathioprine).
Celiac disease can be considered as an atypical form of autoimmune enteropathy. This autoimmune disorder of the small intestine is triggered by the ingestion of gluten-containing food in susceptible individuals. Similar to AIE, intestinal inflammation in celiac disease is characterized by villous atrophy.[11–14] However, celiac disease differs in important points from classical AIE. For example, the colon is not involved in celiac disease unlike in AIE. Celiac disease is also characterized by intra-epithelial T-cell infiltration whereas AIE is marked by infiltration of the lamina propria. Furthermore, celiac disease and AIE differ in the type of T-lymphocytes involved, as indicated by γ/δ T-cell receptor and α/β T-cell receptor, respectively. Most notably, celiac disease is triggered by an exogenous antigen (amino-peptides of gluten). Thus, the enteropathy of celiac disease disappears on a gluten free diet. However, this intervention does not alleviate symptoms in patients with AIE or IPEX syndrome.

1.1.2 Occurence of AIE

To date about a hundred-and-fifty cases of AIE have been published in literature. Table 1 gives an overview of all cases reported until September 2010.

Table 1. Summary of Cases of Autoimmune Enteropathy described in Literature from 1970 untill 2010

families	patient number	sex	Foxp3 mutation	age of onset	death	enteropathy	diabetes	eczema	allergy	trombocytopenia	anemia
1970 Meyer	1.1	m		36 days	4.5 months	yes	yes				
	1.2	m		3 days	32 days		yes				
1977 Dodge	2	m		birth	40 hours		yes				
1982 Ellis	3.1	m		1 week	21.5 months	yes		yes		yes	yes
	3.2	m		birth	11 weeks	yes	yes	yes			yes
1982 Walker-Smith	4.1	m		1.5 year		yes					
	4.2	m		3 weeks	1 year	yes	yes				
1982 Savage	5	m		4 months	16 months	yes	yes				
1982 Hattevig	6	m		8 days		yes	yes				
1982 Powell	7.1	m	A>G(AAUAAA--> AAUGAA)	1 month	10 months	yes	yes	yes			yes
	7.2	m	A>G(AAUAAA--> AAUGAA)	9 months	30 years	yes	yes	yes			yes
	7.3	m	A>G(AAUAAA--> AAUGAA)	childhood		yes		yes			yes
	7.4	m	A>G(AAUAAA--> AAUGAA)	birth	4 years	yes	yes				
	7.5	m	A>G(AAUAAA--> AAUGAA)	birth	3 months	yes	yes				
	7.6	m	A>G(AAUAAA--> AAUGAA)	3 months	25 months	yes					
	7.7	m	A>G(AAUAAA--> AAUGAA)		2 years			yes			
	7.8	m	A>G(AAUAAA--> AAUGAA)	4 months	6 months	yes					
1990 Seidman	8	m		2.5 months		yes	yes				
1991 Jonas	9	m		< 3 year	childhood	yes	yes				
1991 Jonas	10	m		1 year	6 months	yes	yes				
1993 Satake	11.1	m	227 del T	birth		yes	yes				
1998 Kobayashi	11.2	m	227 del T	birth	10 months	yes	yes				
2001 Kobayashi	11.3	m		1 month	6 months	yes					
1995 Roberts	12.1	m	1290--1309 del /ins TGG	2 weeks	10 months	yes	yes	yes			yes
1996 Peake	12.2	m	1290--1309 del /ins TGG	4 weeks	19 months	yes	yes	yes			
2001 Wildin	12.3	m	1290--1309 del /ins TGG	17 days	10 weeks	yes	yes				
	12.4	m	1290--1309 del /ins TGG		childhood	yes					
2000 Clio	13	m		12 hours	26 days	yes	yes	yes			
2000 Chatila	14	m	IVS9+4 A>G	3 weeks-11 months		yes	yes	yes			
	15	m				yes	yes	yes			
2000 Ferguson	15.1	m	1150G>A	1 month		yes		yes			yes
2001 Bennet	15.2	m	1150G>A	2 months	10 months	yes		yes			yes
2006 McGinnes	15.3	m	1150G>A	2 weeks	2 years	yes	yes	yes			yes
	15.4	m	1150G>A	3 weeks	12 weeks		yes	yes			
2001Bennet	16	m	1293-1294 del CT								
1998 Kobayashi	17	m	1087A>G	6 months	3 years	yes	yes				
2001 Kobayashi	17	m		6 months	3 years	yes	yes				
2001 Levy-Lahad	18.1	m	1189C>T	birth	19 days	yes		yes		yes	yes
	18.2	m		birth	5 weeks	yes	yes				
2001 Wildin	18.3	m		birth	5 months	yes	yes				
2001 Wildin	19	m	1113G>T			yes	yes	yes			yes
2001 Wildin	20	m	1150G>A		4 months	yes	yes	yes		yes	
2002 Wildin	21	m	1040G>A	3 months	14 years	yes	yes				
2002 Wildin	22	m	IVS9 + 459 A>G	<1 month		yes	yes	yes			yes
2002 Wildin	23	m	748-750del AAG/ 543C>T	2 months	10years	yes	yes				
2003 Owen	24.1	m	codon 76 de la	2 weeks	6 weeks	yes		yes			
	24.2	m		3 weeks		yes		yes			
2003 Owen	25.1	m		4 months	18 months	yes	yes				
	25.2	m		5 months	19 years	yes		yes			
	25.3	m		17 months		yes	yes				yes
	25.4	m		1 year		yes					
2004 Nieves	26	m	1150G>A	7 months		yes	yes	yes			
2005 Mazzolari	27	m	del promotor 6600pb before start codon	4 months		yes		yes			
2005 Bindl	28	m	IVS8-20 A>C	7 years		yes		yes			
2005 Bindl	29.1	m	g.4859-6247 del	3 weeks		yes		yes			
	29.2	m	g.4859-6247 del	2 months		yes		yes			

Table 1. Summary of Cases of Autoimmune Enteropathy described in Literature from 1970 untill 2010

families	patient number	sex	Foxp3 mutation	age of onset	death	enteropathy	diabetes	eczema	allergy	trombocytopenia	anemia
2007 Torgerson	29.1	m	g.4859-6247 del	3 weeks		yes		yes			
	29.2	m	g.4859-6247 del	2 months		yes		yes			
2010 Moes	29.1	m	g.4859-6247 del	3 weeks		yes		yes			
	29.2	m	g.4859-6247 del	2 months		yes		yes			
2006 Bachetta	30	m	1305-1306 TT>GC/543C>T	2 weeks		yes	yes	yes			yes
2006 Bachetta	31	m	970T>C	4 months		yes		yes			
2006 Bachetta	32	m	3G>A	birth		yes	yes	yes			
2006 Meyers	33	m	1-7G>T	1 day	54 days	yes	yes				
2006 Meyers	34	m	1169G>A	4 days	1 year	yes	yes	yes			yes
2006 benedetti	35	m	454+4 A>G	18 days		yes		yes			
2006 Benedetti	36	m	323C>T	14 months		yes					
2007 Mc Lucas	37.1	m		< 1 year		yes		yes			yes
2007 Lucas	37.2	m		3 months		yes					
2007 Rao	38	m	IVS9 A>G			yes	yes	yes			yes
2007 Rao	39	m	303-304 del TT	4 months		yes	yes				yes
2007 Moudgil	39	m		4 months		yes	yes	yes			yes
2007 Rao	40	m	1271G>A			yes	yes	yes			
2007 Rao	41	m	1226A>G			yes	yes				
2007 Taddio	42	m	1150G>A	1 month		yes	yes	yes			
2007 Suzuki	43	m	1099T>C	8 days	16 weeks	yes	yes	yes			
2007 Zuber	44	f		10 years		yes					yes
2008 Gambineri	45	m	2T>C	neonatal	3 months	yes	yes				
2008 Gambineri	46	m	3G>A	neonatal		yes	yes	yes			
2008 Gambineri	47	m	IVS1+T2>G	neonatal		yes	yes	yes			
2008 Gambineri	48	m	543C>T	neonatal	5 months	yes	yes				
2008 Gambineri	49	m	543C>T; F324L	neonatal		yes	yes	yes			
2008 Gambineri	50	m	IVS7+5G>A	neonatal	9 months	yes	yes	yes			
2008 Gambineri	51	m	IVS8+4A>G	neonatal		yes	yes	yes			
2008 Gambineri	52	m	P339A FKH domain	neonatal	6 months	yes	yes				
2008 Gambineri	53	m	R347H FKH domain	neonatal		yes	yes	yes			yes
2008 Gambineri	54	m	R347H FKH domain	< 1 year		yes	yes				
2008 Gambineri	55	m	A384T FKH domain	neonatal		yes	yes	yes			
2008 Gambineri	56	m	F373A FKH domain	neonatal		yes	yes	yes			
2008 Gambineri	57	m	F374C FKH domain	neonatal	11 months	yes		yes			
2008 Gambineri	58	m	L242P	4 months		yes		yes			
2009 Rubio-Cabezas	59.1	m	V408M	3 weeks		yes	yes				
	59.2	m	V408M	3.5 months		yes	yes				yes
2009 Rubio-Cabezas	60	m	V408M	2 days		yes	yes				
2009 Rubio-Cabezas	61	m	R337Q	30 days	13 months	yes	yes	yes			
2009 Rubio-Cabezas	62	m	P339A	1 week	5.5 months	yes	yes			yes	yes
2009 Rubio-Cabezas	63	m	L76QfsX53	1 day	8 months	yes	yes				
2009 Hashimura	64	m		2 months			yes				
2009 Mhoko	65.1	m		1 months				yes			
	65.2					yes		yes			
	65.3							yes			
	65.4							yes			
2009 Scallon	66	m		8 month		yes	yes			yes	
2010 Moes	67	m	560 c>t	birth	8 years	yes		yes		yes	yes
2010 Moes	68	m	g.1121 t>g	birth	14 months	yes		yes			yes
2010 Moes	69	m	751-753 del gag	6 weeks		yes		yes			yes
2010 Moes	70	m	751-753 del gag	4 weeks	8 months	yes	yes	yes			yes
2010 Moes	71	m	c. 1015 c>g	7 days	7 months	yes	yes	yes			yes
2010 Moes	72	m		4 weeks		yes	yes	yes			yes
2010 Moes	73	m		4 months		yes					
2010 Moes	74	f		4 months		yes		yes			
2010 Moes	75	f		3 months		yes		yes		yes	yes

Table 1. Overview of cases of autoimmune enteropathy published in literature until September 2010. Cases are listed by author and year of publication. Patients are numbered by family followed by an individual number (for example 1.1 = patient from family 1, first individual).

1.1.3 Pathophysiology of AIE

First insight into the pathogenesis of AIE came from a study by Powell et al. that was published in 1982.[15]

In this study, the authors described 17 boys over three generations of one family with various autoimmune disorders, including a severe form of AIE. This observation suggested a genetic, X-linked mode of transmission. The molecular basis of the disease was further aided by the discovery of the genetic basis in scurfy mice. Scurfy mice and boys with X-linked AIE share important clinical similarities. The naturally occurring scurfy mice develop a severe and rapidly lethal autoimmune syndrome with a massive infiltration of liver, lung, pancreas, skin and gut, but no diabetes mellitus. In these mice, massive and simultaneous type 1 and type 2 T-helper lymphocyte responses are observed. The loss of function mutation within the *scurfy/Foxp3*-gene results in a complete absence of regulatory T-cells in homozygous male animals.[16]

While there are several differences in Forkhead Box Protein 3 (FOXP3) expression and function between mice and humans[17], phenotypic resemblance led to the discovery of disease-causing mutations in the *FOXP3*-gene of patients with IPEX syndrome. This finding represents an enormous step forward towards the understanding of AIE.[18]

The connection between severe autoimmune symptoms and an absence of regulatory T-cells was confirmed with the development of a *Foxp3*-/- knockout (KO) mouse-model. These KO mice completely lack regulatory T-cells (Tregs) and share an identical phenotype as the scurfy mice.[19] In both animal models, introduction of Foxp3+ cells reconstitutes regulatory T-cells.[19,20] In subsequent studies, principally performed in mice, Foxp3/ FOXP3 is identified as a key transcription factor that is indispensable for regulatory functions of T-cells.[19,21] These studies indicated a critical role of regulatory T-cell homeostasis in the development of AIE.

The molecular mechanisms of *FOXP3*-independent forms of AIE are currently under investigation. An interesting molecular candidate implicated in the pathogenesis of *FOXP3*-independent AIE is IL-2 and its high affinity receptor CD25. Caudy et al.[22] recently described a patient with an IPEX-like clinical presentation with completely normal *FOXP3*-gene but marked CD25 deficiency on $CD4^+$T-cells, which was due to an autosomal recessive mutation in the interleukin-2 (IL-2) receptor gene. This preliminary finding suggests that molecules other than FOXP3 can be implicated in the regulation, function and/or homeostasis of regulatory and/or effector T-cells.

1.1.4 Molecular background of AIE

FOXP3

The *FOXP3*-gene consists of 11 coding exons and a non-coding part upstream of exon 1.[19,20] In IPEX syndrome mutations are described in all regions with most occurring in the Forkhead

Domain (exon 9–11).[3,23]

The *FOXP3*-gene encodes a 48kDa protein of the forkhead (FKH)/winged-helix transcription factor family, named FOXP3 or scurfin. The protein can be divided into 5 main regions: the N-terminus, the Zinc Finger Domain, the Leucine zipper, the Forkhead Domain and the C-terminus.[24] Recently, Ziegler and colleagues attached nuclear localization and repressor function to the forkhead domain of the protein, and a role in the homodimerization to the leucine zipper. They further described the N-terminal domain of the FOXP3-protein to be required for FOXP3-mediated repression of transcription from both a constitutive active- and a NF-AT(nuclear factor of activated T-cells)-inducible promotor.[24] *FOXP3*-gene and FOXP3-protein are represented in Figure 1.

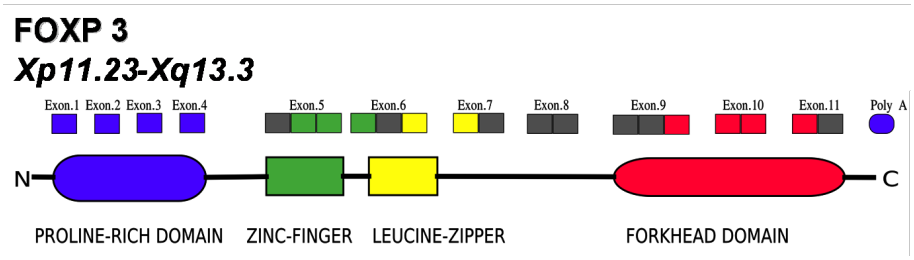

Figure 1. *FOXP3*-gene with 11 exons and a poly A part. Underneath, the representation of the FOXP3-protein with its different functional domains. The exons are painted in the same colour as the protein domains they encode.

The FOXP3 protein is expressed at high levels in $CD4^+CD25^+$ regulatory T-cells[19] and is considered the major determinant for a T-cell to acquire regulatory functions.[17]

The precise mechanisms of FOXP3 function are only partially elucidated. FOXP3 binds to DNA via the forkhead domain and functions as a transcriptional repressor.[21] Forkhead binding domains were identified to be adjacent to NF-AT transcription factor binding sites in the promoters of several cytokine genes, including IL-2 and tumor necrosis factor (TNF). This led to the proposal of a model of *FOXP3*-mediated transcriptional repression in which FOXP3 antagonizes NF-AT function by competition for DNA binding sites.[25–27] Another suggestion is that FOXP3 may inhibit NF-AT-activity independent of NF-AT binding. One possible mechanism is through the recruitment of transcriptional co-repressors to the promotors of NF-AT regulated target genes, resulting in inhibition of the transcriptional machinery.[24] However, so far, the characterization of FOXP3 target genes or the transcriptional program specified by FOXP3 is only partially known. Two recently published studies suggested that FOXP3 is capable of recruiting the chromatin remodeling machinery to its binding site as a mechanism of regulating gene expression. Thus, it can regulate as many as 300 distinct genes.[28,29]

T-cell Immunity

T-lymphocytes are important players in adaptive immunity. These cells are thymus derived and possess a T-cell antigen receptor, which can be of α/β or γ/δ type. They also express co-receptors CD4 or CD8. T-lymphocytes have an enormous variability in antigen recognition with high specificity, potent effector function and long lasting immunological memory.[30]
Subsets of T-lymphocytes include T-effector cells and regulatory T-cells.[30]
T-effector cells actively participate in the immune reaction upon activation by peptic antigen that is presented on Major Histocompatibility Complex (MHC) molecules expressed on Antigen Presenting Cells (APC).[30]
Subsequently, $CD8^+$-cytotoxic T-cells attack the antigen through a mechanism of cytotoxic killing. $CD4^+$ T-helper cells either stimulate B-cells to proliferate, differentiate and produce antibody or activate macrophages. Once T-helper cells are activated, they divide rapidly and release small proteins called cytokines that regulate or assist in the active immune response.[30]
Activated T-effector cells perform the principal function of the immune system, which is to protect the host from pathogenic microorganisms. However, their actions can also become harmful to the host when the immune reaction is excessive or directed against self antigens.[30]
Mechanisms to control the proper regulation of T-cell function include immunological self-tolerance (i.e. making T-cells unresponsive to self antigens) and homeostatic equilibrium (i.e. controlling the magnitude of the T-cell response to foreign antigens in order to achieve a steady state situation of the immune system). Maintenance of homeostatic equilibrium is achieved through different mechanisms that include regulation of T-cell death and development through positive and negative selection of T-cells in the thymus.[31] Regulatory T-cells have a major role in the maintenance of tolerance and homeostasis through cell-contact or cytokine-dependent mechanisms on T-effector cells, resulting in down-regulation of T-effector cell activation and function. Regulatory T-cells shut down T-effector cell mediated immunity towards the end of the immune response against foreign antigens (Figure 2). Tregs also prevent immunological reactions to self. When Tregs are dysfunctional or absent, the immunological response to foreign antigen is exaggerated and the immune system can attack self tissues. This leads to severe inflammatory reactions and autoimmune disease as seen in AIE.[31–34]

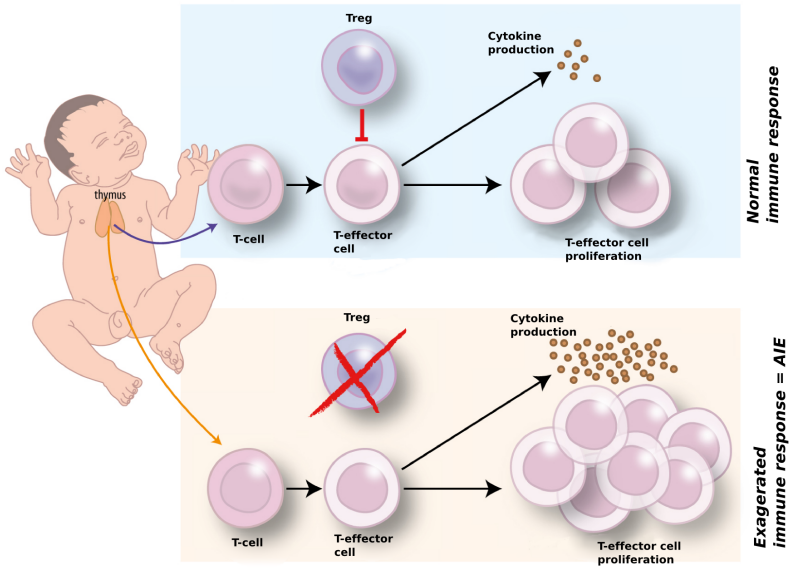

Figure 2. $CD4^+CD25^+FOXP3^+$ Tregs block the activation and expansion of effector T-cells thereby suppressing the immune response. When these cells are non-functional-as in autoimmune enteropathy, the immune response is exaggerated. Image courtesy the NTvG [35]

Regulatory T-cell characteristics

Based on studies in mice, regulatory T-cells can be classified into three subsets according to mechanism of T-effector cell activation (e.g. soluble factors or cell-contact mediated) and site of origin (e.g. thymus or peripheral lympoid organs). There are two types of inducible regulatory T-cells: Tr1 and Th3 cells. These cells are generated in the peripheral lymphoid organs. Tr1 cells and Th3 cells produce cytokines IL-10 and TGF-β, respectively, to suppress T-effector cell activation.[32,36–38]

Natural Treg cells are $CD4^+$ regulatory T-cells that were generated in the thymus in a *FOXP3*-dependent manner.[37,39] The exact molecular mechanisms of their differentiation and the precise role of FOXP3 are not completely understood. IL-2 is thought to be necessary in the generation of lymphocytes in the thymus and maintenance of homeostasis in the periphery. CD28 is also needed, most likely involved in the regulation of IL-2 production by conventional T-lymphocytes and CD25 expression regulatory T-cells.[32]

Natural Tregs represent 5-10 % of the total $CD4^+$ T-cell population in mice and $< 1\%$ in human peripheral blood.[32] While natural Tregs suppress T-effectors cells by cell-contact dependent mechanisms in mice, recent studies have suggested that more heterogeneous mechanisms are involved in humans. There is evidence to support possible $CD4^+CD25^+FOXP3^+$ T-cell development in the periphery as well as influence of several cytokines on $CD4^+CD25^+FOXP3^+$

function.[32]

A remaining issue with Treg cells relates to difficulties with phenotype characterization as there is no specific Treg marker. Treg cells belong to the subgroup of $CD4^+$ cells with high expression of CD25. However, conventional non-Treg $CD4^+$ cells also transiently express CD25 after activation. Thus, the mere presence of CD25 is not enough. Characterization of Treg cells also require high expression levels of the protein. Therefore, $CD4^+$ T-cells are divided in $CD25^-$, $CD25^{intermediate}$ and $CD25^{high}$ subsets with Treg cells belonging to the last subset. Recently it has been proposed to further subdivide the $CD25^{high}$ group into $CD25^{++}$ and $CD25^{+++}$ subsets. Under this classification scheme, Treg cells belong to the latter group.[40]

Additionally, other phenotype markers such as CD2, CTLA-4, GITR, and integrin CD103, which are found on the membrane of Treg cells, are not specific identifiers of these cells.[41,42] These markers are also expressed by activated T-cells. It has been demonstrated recently that CD127 (IL-7Rα) expression is down-regulated on $CD4^+CD25^+$ regulatory T-cells. $CD4^+CD25^+$ cells that have down-regulated the expression of CD127 are anergic and show suppressive activity in vitro.[43] To date, however, the most specific marker for regulatory T-cells is the transcription factor FOXP3.[23] Transfection of this factor into naive $CD4^+$ T-cells induces a phenotype and function that are consistent with regulatory T-cells.[23]

$CD4^+CD25^+$high Treg cells play a critical role in maintaining immunological self tolerance by actively suppressing self reactive lymphocytes.[31] This explains the occurrence of various autoimmune phenomena in AIE. However, recent findings suggest that these cells can also react to foreign antigens. The important gastrointestinal and skin symptoms of some forms of AIE support this idea, as these sites are interfaces of the body where they are exposed to exogenous antigens such as alimentary antigens or bacterial products.

The suppressive functions of Treg cells are thought to be dependent on cell-cell contacts[44] and not mediated via soluble immunosuppressive cytokines. However, some in vitro and in vivo studies suggest that TGF-β and IL-10 may be important mediators involved in Tregs functions.[45] Another recently discovered cytokine, named IL-35 (Ebi3/IL-12α heterodimer), is thought to be constitutively secreted by Tregs and not by T-effector cells. Epstein Barr virus-induced gene 3 (Ebi3, which encodes IL-27β) and interleukin12 alpha (which encodes IL-12α/p35) are highly expressed by mouse Treg cells but not on resting or activated T-effector cells. Treg-cells restriction of the cytokine occurs because Ebi3 is a downstream target of FOXP3.[46,47] The role of this cytokine secretion by Treg cells is to date unclear.

The Vitamin A metabolite Retinoic Acid (RA) has recently been identified as a co-factor in peripheral Tregs cell regulation. Several groups reported that RA induces gut imprinting and enhances Tregs conversion, differentiation and expansion when preserving regulatory T-cell suppression.[48,49]

It is known that natural Tregs express high amounts of folate receptor 4. Administration of

monoclonal antibody to folate receptor 4 specifically reduced Tregs cells and elicited autoimmune disease in mice.[50]

T-helper cell characteristics

T-helper subsets

Classically, T-helper cells (Th-cells) are separated into three lineages (Th-1, Th-2 and Th-17) according to their restricted and stable pattern of cytokines. Th-1 cells are characterized by the production and secretion of interferon-gamma (IFN-γ), whereas Th-2 cells produce IL-4, IL-5, and IL-13. In mice, Th-1 cell-mediated responses are cellular responses that are characterized by activation of T-cells and macrophages and production of interferon-gamma and IgG2a. In contrast, Th-2 cells provide support for humoral responses through IgG1 and IgE production, sensitization of mast cells and eosinophil maturation.[51] The Th-17 subset produces interleukin IL-17 and seems to be involved in various models of immune-mediated tissue injury, including 1) organ-specific autoimmunity in the brain, heart, synovium and intestines, 2) allergic disorders of the lung and skin, and 3) microbial infections of the intestines and the nervous system.[52]

More recent experimental data, however, argue against fixed characterization into lineages as the evidence suggests great plasticity of $CD4^+$ T-cells (i.e. they can switch from one subset to another according to different environments/situations).[53]

Figure 3 represents these two different views on T-helper cell characteristics.

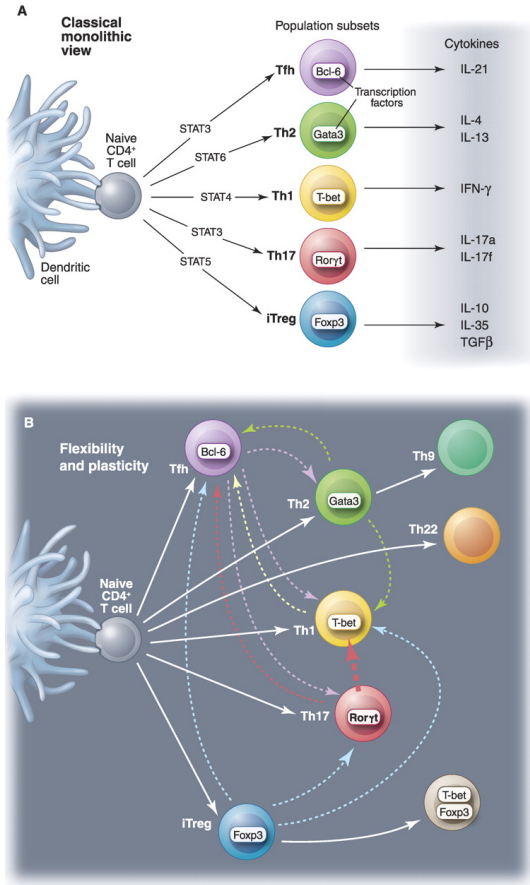

Figure 3. T-helper cell differentiation. From Th-0 cells arise Th-1, Th-2, Th-3 and Th-17 cells. Panel A represents T-helper-cell subsets as different lineages and panel B as the the nowadays more accepted plastic model. Image courtesy Science.[53]

T-helper cell activation

T-helper cells express CD4 and use this molecule as a co-receptor in association with their antigen-specific T-cell receptor (TCR) to recognize antigens. TCRs are generated by means of gene-segment recombination, leading to TCR polymorphism. The antigen is bound to major histocompatibility complex class II (MHCII) present on antigen presenting cells (APC) and B-cells and consists of enzymatically cleaved peptide fragments (8-10 amino acids) of protein antigen.[30] A first signal for activation of the Th-cells with the correct specificity is provided by the recognition of this MHCII-peptide complex via the TCR-CD3 complex. Recognition of a specific epitope by an effector T-helper cell (Th-cell) induces the expression of CD40-ligand and CD28 on the T-cell surface. Binding of this CD28 molecule to either B7-1 (CD80) or

B7-2 (CD86) present on APC generates a signal 2 for T-cell activation. The activated Th-cell on its turn can provide signal 2 for cytotoxic T-cells (Tc) and B-cells, and produces immunomodulating-cytokines such as interleukin (IL) 2. The signal 3 of T-cell activation is mediated by cytokines such as IL-2. This signal is independent of signal 1 and 2 and potently influences cell cycle progression. The 3 signals of T-cell activation are represented in Figure 4.[54,55]

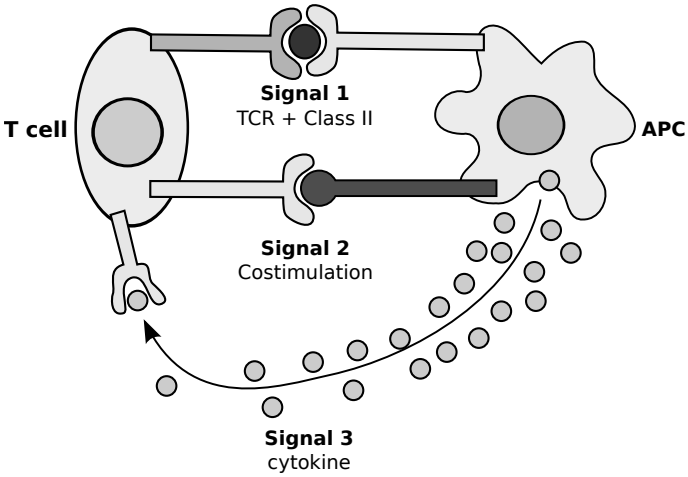

Figure 4. Mechanisms of T-cell activation. Signal 1 passes via the T-cell receptor. This signal is co-stimulated by signal 2 that passes via CD28/CD40 molecules. Together they act on the calcineurin pathway of gene transcription. Signal 3 is dependent on different cytokines such as IL-2. This signal influences cell cycle progression.

T-effector cell function in AIE

Bacchetta et al.[56] recently observed that in some IPEX patients beside abnormal Treg-suppressor functions, also T-effector-cell functions were comprised, indicating a more general T-cell defect. Upon CD3 and CD28 stimulation, $CD4^+$ effector T-cells of IPEX patients produced markedly less IFN-γ and IL-2 compared to healthy control T-cells. As IL-2 is a master cytokine for regulatory T-cell functions, this defect may further aggravate already insufficient regulatory T-cell controls. However, these findings await confirmation.

1.1.5 Diagnosis of AIE

Gastroenterological exams:

Clinical

Diarrhoea in AIE is abundant with volumes up to 100 mL/kg/day. Despite bowel rest, diarrhoea persists and shows signs of protein loss in feces. This subsequently leads to massive hypoalbuminea. Chronic inflammation leads to elevated levels of fecal calprotectin and blood loss. However, these are not specific findings for AIE. Differentiating of AIE from other inflammatory bowel diseases is based on endoscopic and immunologic results.[14]

Endoscopy

Macroscopic lesions of the small bowel range from minimal erosions, large erythematous lesions to deep ulcerations. In the colon a loss of the normal vascularization pattern and edema is always present. In addition, ulcerations are often seen.

Histology

Biopsies of AIE patients show severe villous atrophy of small bowel. Important infiltration of the lamina propria by lymphocytes is typically seen, and tissue destruction with villous atrophy, increased apoptosis and destruction of the epithelium are observed.[14] (Figure 5ab) Additionally, ulcerations and a more polymorph inflammatory infiltrate is possible.

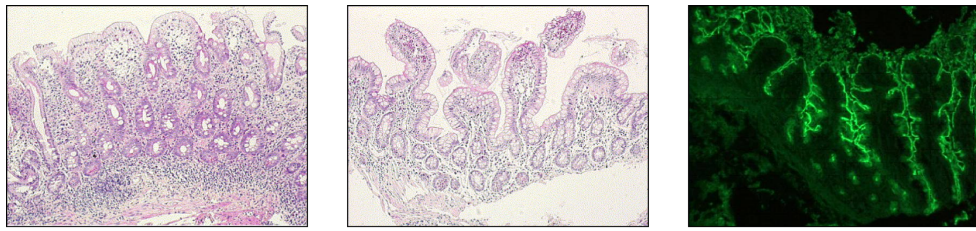

Figure 5. Duodenal biopsy with autoimmune enteropathy, showing the dense mononuclear infiltrate of the lamina propria. Note the marked villous atrophy and differentiation of the intestinal epitelial cells. (A) is 10x enlarged and (B) is 25x enlarged. (C) immunofluorescence staining pattern of circulating anti-enterocyte antibodies, which are directed against the brush border membrane of intestinal epithelial cells. Image courtesy of J Pediatr and Walker WA et al. Pediatric Gastrointestinal Disease. [57] [14]

Immunological tests:

auto-antibodies

Patients with AIE (*FOXP3*-dependent or independent) generate a variety of autoantibodies. Therefore, a major characteristic and essential element in the diagnosis of autoimmune entero-

pathies is the presence of auto-antibodies against human enterocytes or colonocytes. These are mainly antibodies of IgG subclass. Immunofluorescence studies showed that these auto-antibodies are directed against components of the intestinal brush border membrane, with an increasing intensity from crypts towards villus tip.[58] (Figure 5c) 'Anti-enterocyte antibodies' at low titers were also described in other inflammatory gut conditions such as Crohn's disease, ulcerative colitis and cow's milk allergy.[59,60] A pathogenic role of these antibodies in the onset of intestinal inflammation – as suspected in the past – is unlikely.[58] Neither is there a correlation between these antibodies and severity of the mucous lesions. Anti-AIE-75 are highly specific antibodies for IPEX disease. [14,58,61]

Other laboratory results

Inflammation markers as CRP and sedimentation rate are often elevated in AIE. In some children signs of coombs positive autoimmune anemia or thrombocythopenia are found.

Immunological testing of IPEX patients shows normal peripheral blood counts and subsets of $CD3^+$ (90%), $CD4^+$ (60-65%), $CD8^+$ (20-30%) and $CD19^+$ cells (5%), in some patients with slightly reduced T-cells with NK marker CD56. The patients express normal levels of IgM and IgA. IgG levels are higher than normal. In IPEX typically IgE levels are strikingly elevated (range between 2000 and 4000 IU/ml).[2] IPEX patients show low absolute numbers of FOXP3-positive cells but express the same level of $CD4^+CD25^+$ cells as healthy controls. An increased number of activated T-cells CD4CD25 reflect the massive inflammatory and immunostimulatory response. However in functional assays these $CD4^+CD25^+$ regulatory T-cells do not show suppressor function.[2]

Immunological testing of patients with *FOXP3*-independent autoimmune enteropathy have normal T-cell subsets and normal antibody levels. There are elevated levels of IgE, but in general the levels are lower than in IPEX patients (500-1000 IU/ml). Patients may have no *FOXP3*-mutations but can show a lower than normal level of FOXP3-mRNA and protein. $CD4^+CD25^+$ T-cells are present in normal to higher levels than healthy controls and suppressor function of these cells is disturbed.[2]

Genetic tests

Genetic tests are performed to show mutations in the *FOXP3*-gene, confirming the diagnosis of IPEX syndrome.[14]

1.1.6 Treatment of AIE

Immunosuppression

Treatment of AIE and particularly IPEX syndrome is extremely challenging.[3,4] In the initial phase of the disease, all patients are highly dependent on parenteral nutrition to achieve primary stabilization. Different treatment strategies for AIE were attempted in the past. However, to date only two approaches seem to be successful: immunomodulation and hematopoietic stem cell transplantation (HSCT).[1,3,4,62] Some extremely positive results were gained in treating AIE patients with steroids alone or in combination with azathioprine, cyclosporin A (CsA) or tacrolimus.[63–66] Other approaches include the use of immunoglobulins, anti-lymphocytic immunoglobulin or cyclophosphamide, which were all rather inefficient.[67] A recent study documented the successful use of anti-tumor necrosis factor (TNF) antibodies in the treatment of a patient with AIE.[68] However, upon three injections, the treatment was stopped and high dose-steroid medication was again required to maintain remission. 'Necker hospital' reported that only some children with AIE respond to anti-TNF therapy in a sustained manner (unpublished data).

In general, AIE that is not related to mutations in *FOXP3* responds well to corticosteroid therapy combined with azathioprine.

The best results with immunomodulators in IPEX syndrome were obtained with tacrolimus, whereas only very few patients respond to CsA.[4,66] Likewise, T-cells from scurfy mice are highly resistant to CsA suppression, indicating that agents inhibiting TCR signaling may be of limited benefit.[16] Furthermore, tacrolimus has severe side-effects such as renal toxicity and some patients do not improve on the drug. Therefore, rapamycin was recently introduced as an alternative immunosuppressor. The results to date are promising with responses that seem to be as good as tacrolimus but with fewer side effects.

Tacrolimus

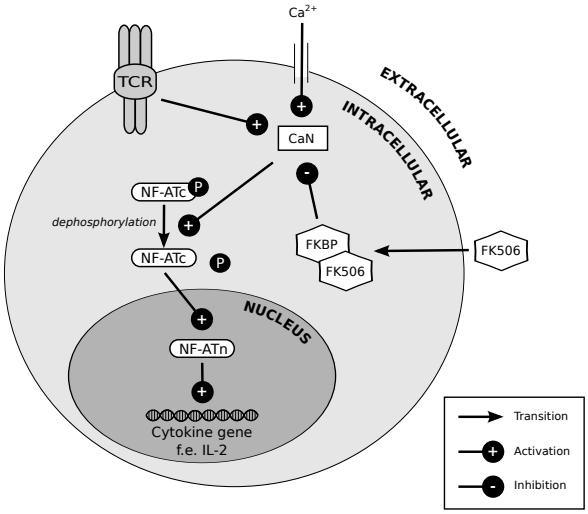

Figure 6. Mechanism of action of tacrolimus (FK506, Prograf®).

Tacrolimus (FK506, Prograf®) is a calcineurin-dependent immunosuppressor that potently inhibits signal 1 and 2 of T-cell activation via the T-cell receptor and co-stimulation through CD28. Tacrolimus binds to the cytoplasmic FK506-binding protein (FKBP) to form a FK506-FKBP complex, which then binds to calcineurin (CaN) and block its biological activity. As a result, calcineurin fails to dephosphorylate the cytoplasmic component of the nuclear factor of activated T-cells (NF-AT). This inhibits the nuclear translocation of cytoplasmic (NF-ATc) and subsequent binding to the nuclear component of the nuclear factor of activated T-cells (NF-ATn). Normally, the NF-ATc–NF-ATn complex binds to the promotor of the interleukin 2 (IL-2) gene and initiates IL-2-transcription. When this process is blocked by tacrolimus, T-cells are not able to produce IL-2, which is necessary for full T-cell activation. (Figure 6)
69

Rapamycin

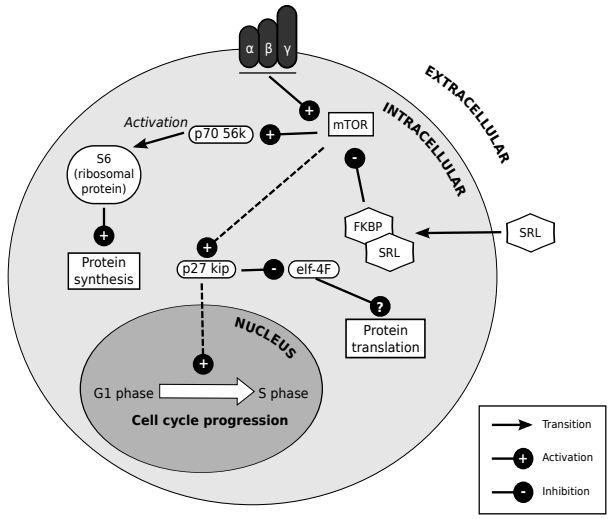

Figure 7. Mechanism of action of sirolimus (Rapamycin).

Rapamycin is a calcineurin-independent immunosuppressor. Its functional molecule sirolimus (SRL) binds to the same FK506-binding protein (FKBP) as tacrolimus. However, the SRL-FKBP complex does not target calcineurin but rather the mammalian target of Rapamycin (mTOR). The SRL-FKBP-mTOR complex mediates inhibition on signal 3 of T-cell activation through the cytokine receptor and other targets such as the 70-kD S6 protein kinase p70S6 and the eukaryotic initiation factor (EIF). As result, the complex influences biochemical pathways that are required for cell progression through the late G1 phase or entry into the S phase of the cell cycle. (Figure 7).[70]

Hematopoietic Stem Cell Transplantation (HSCT)

Many IPEX patients do not respond to immunomodulation with a sustained control of disease. Given the serious side effects, toxicity and limited efficacy for long-term remission with immunosuppressive medication, there is a major need for new therapeutic approaches. Currently, HSCT is the only possible way to cure IPEX syndrome. The technique is now established in some specialized centers. In the past, survival was poor with HSCT, specifically during myeloablative therapy in the pre-transplantation phase.[62] Survival has improved with current adapted protocols and patients with follow up for more than 4 years have been reported.[8–10] However, IPEX syndrome patients remain very difficult to transplant.[3,4]

1.2 Rationale

Until recently, the molecular basis of autoimmune enteropathy remained largely undefined. The recent discovery of disease causing mutations in *FOXP3* leading to IPEX syndrome pointed to a crucial role of regulatory T-cells in the pathophysiology of this type of AIE. Clinical presentations of IPEX-like and other *FOXP3*-independent forms of AIE suggest other mechanisms of autoimmune inflammation. Other transcription factors or regulators of FOXP3 may play a role in these IPEX-like forms of AIE. More investigations are needed to explain the molecular basis of these distinct forms of AIE. There is a wide range of factors influencing regulatory T-cell function. The resulting complexity of immunologic homeostasis opens doors for different diagnostic tools and therapeutic possibilities. Currently, immunosuppressors and HSCT are the only treatment options. However, results with HSCT are not consistent. Therefore, new immunosuppressive strategies in the treatment of IPEX disease are needed.

1.3 Aim

The main aim of this work is to precisely describe the different phenotypes of AIE and to gain further insight into the molecular background of the disease.
Specific aims are:
1) to address and study the function of regulatory T-cells in autoimmune enteropathy.
2) to analyze the clinical phenotype of patients relied to mutations in *FOXP3*, and the hypothesis of a potential genotype-phenotype correlation in IPEX.
3) to study the mechanisms of immunosuppressive therapy in AIE.

1.4 Outline

The studies concerning the same specific aim are bundled together to form a chapter in this thesis. Chapter two focusses on regulatory T-cell function. In the past it was believed that natural regulatory T-cell function was solely dependent on cell contact with T-effector cells. More recently it was reported that environmental factors, like cytokines IL-2, TGF-β and IL-10 could influence Treg function. We performed an *in vitro* study to analyse the effect of intestinal pro-inflammatory cytokine IL-15 on Treg function. In chapter three we describe a new phenotype of IPEX syndrome in which the enteropathy is combined to severe immunoallergic reactions. This phenotype resulted from a mutation in the *FOXP3*-gene that was thus far not known. We hypothized a possible genotype-phenotype relationship and performed a study in 8 patients with IPEX to test our hypothesis. Furthermore we analyzed for the first time patients with *FOXP3*-independent AIE. Current treatment of severe forms of AIE is based on T-cell immunosuppression. In chapter 4 we describe a rare but severe side effect of tacrolimus treatment. Secondly, in an *in vitro* study we compare the molecular effect of tacrolimus and rapamycin on IPEX cells.

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CHAPTER 2

REGULATORY T-CELL FUNCTION IN AIE

IL-15 Renders Conventional Lymphocytes Resistant to Suppressive Functions of Regulatory T-Cells through Activation of the Phosphatidylinositol 3-Kinase Pathway

Mélika Ben Ahmed, Nadia Belhadj Hmida, Nicolette Moes, Sophie Buyse, Maha Abdeladhim, Hechmi Louzir and Nadine Cerf-Bensussan.

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Abstract

IL-15 drives chronic inflammation in several human diseases. We have recently shown that IL-15 inhibits the immunosuppressive effects of TGF-β through blockage of the Smad3-signaling pathway. Data pointing to reciprocal interactions between TGF-β and CD4(+) regulatory T-cells led us to investigate the impact of IL-15 on the de novo generation and function of regulatory T-cells in humans. Our data indicate that IL-15 does not counteract, but rather promotes the effect of TGF-β on the de novo generation of regulatory T-cells (Treg). Thus, in the presence of TGF-β, IL-15 enhanced the acquisition of regulatory functions by CD4(+)CD25(-) T-cells stimulated by anti-CD3 and anti-CD28 Abs. In contrast, IL-15 impaired the functions of Tregs by acting on effector CD4 and CD8 T-cells. Accordingly, in the presence of IL-15, proliferation and IFN-γ production by peripheral CD4 and CD8 T-cells could not be efficiently inhibited by Tregs. IL-15-induced resistance of effector T-cells to Tregs resulted from activation of the PI3K signaling pathway but did not involve the rescue of effector T-cells from apoptosis. Altogether, these data point to the ambiguous role of IL-15 in the control of Treg functions. This dual role may be instrumental to mount rapid but transient pro-inflammatory immune responses against pathogens but may become deleterious in situations associated with protracted IL-15 over-expression.

2.1.1 Introduction

Interleukin-15 plays pleiotropic functions at the interface between innate and adaptive immunity and participates both in homeostatic and pro-inflammatory regulation of the immune system. IL-15 is critical for the differentiation and/or survival of several immune cell subsets, including NK cells, memory CD8 T-cells, and intraepithelial lymphocytes. IL-15 is also a potent inducer of the effector phase of the immune response, notably by stimulating dendritic cell maturation as well as NK and CD8 T-cell cytotoxicity (1, 2). We have recently shown that IL-15 impairs the immunosuppressive effects of TGF-β through inhibition of the Smad3 signaling pathway (3), which plays a key role in tipping the immune balance toward

tolerance (4, 5). Because IL-15 is secreted early in response to intracellular pathogens (6), its inhibitory effect on TGF-β-mediated regulation may further promote protective effector immune responses. Conversely, persistent expression of IL-15, observed in several autoimmune or chronic inflammatory diseases (7), may durably impair the regulatory functions of TGF-β and thereby create a vicious circle perpetuating inflammation. TGF-β plays a pivotal role in maintaining immune homeostasis via the direct control of activation, proliferation, differentiation, and survival of immune T-cells but also via its effects on regulatory T-cells (Treg)4 (8, 9). Although not required for the thymic development of natural occurring $CD4^+CD25^+FOXP3^+$ Treg, TGF-β helps the in vitro and in vivo de novo generation of induced Treg from naive $CD4^+CD25^-$ T-cells (reviewed in Ref. 10 and 11). TGF-β is necessary in the periphery for the maintenance of murine Treg and sustains their suppressor functions and FOXP3 expression (12, 13, 14). The direct contribution of TGF-β in their suppressive functions remains, however, controversial. In most in vitro experiments TGF-β is not essential for the suppressor functions of Treg (15, 16). Yet, a role of membrane-bound TGF-β has been occasionally reported (17). Data obtained in vivo are more contentious. Antagonizing TGF-β abolished the therapeutic effect of murine Treg in some but not all in vivo models (18, 19, 20, 21). The role of Treg-derived TGF-β was important in one model implicating induced Treg (20) but not in other models depending on naturally occurring Treg where functional TGF-β was produced by another cellular source (19, 21). Notably, TGF-β was necessary to stimulate the production of IL-10 by the subset of Treg present in the intestine (22). Our recent demonstration that IL-15 exerts an inhibitory effect on TGF-β signaling led us to investigate the impact of IL-15 on the de novo generation and/or function of $CD4^+CD25^+$ Tregs.

2.1.2 Materials and Methods

Patients and samples

Peripheral samples were from healthy volunteers donors. Informed consent was obtained from all patients before the study. All experiments were approved by local ethics committee.

Culture medium and reagents

In all in vitro assays, cells were cultured in RPMI 1640 medium supplemented with 10% AB human serum (Sigma-Aldrich), 1% sodium pyruvate, 1% non essential amino acids, 1% HEPES buffer, 5×10^{-5} M β-ME, and 40 μg/ml gentamicin (Invitrogen). Purified recombinant human IL-15, IL-2, and TGF-β (R&D Systems) and mAbs anti-human CD3 (UCHT1) and CD28 (BD Biosciences) were used for in vitro lymphocyte culture. The following inhibitors of signaling pathways were used: JAK3 inhibitor I (Calbiochem) at 100 μM, inhibitor of JNK (SP600125; BIOMOL International) at 10 μM, inhibitor of p38 MAPK

(SB203580; Calbiochem) at 1 μM and inhibitors of PI3K/Akt pathway, Wortmaninn (Sigma-Aldrich) at 0.5 μM, and Ly294002 (Sigma-Aldrich) at 20 μM. The following mAbs were used for flow cytometry analysis: FITC- and Cy-Chrome-conjugated anti-human CD3, CD4, and CD8 (BD Biosciences); PE-conjugated anti-human CD45RA, CD45RO, phospho-Akt (pT308) (BD Biosciences); anti-human CD25 (Miltenyi Biotec); and anti-human FOXP3 (FOXY, eBiosciences or 259D/C7, BD Biosciences).

Lymphocyte isolation and cell culture

PBMC were isolated on Ficoll-Hypaque gradient and lymphocyte subsets were separated using magnetic beads (CD3, CD8, and anti-PE microbeads, and $CD4^+CD25^+$ Regulatory T-cell Isolation Kit, Miltenyi Biotec). For $CD3^+$, $CD8^+$, $CD4^+CD25^-$, $CD4^+CD45RA$, $CD4^+CD45RO$, $CD8^+CD45RA$, and $CD8^+CD45RO$ isolated subsets, purity ranged from 90 to 98%. $CD4^+CD25^+$ Tregs were collected with a purity ranging from 67 to 84%. For de novo generation of regulatory T-lymphocytes, purified $CD4^+CD25^-$ peripheral T-lymphocytes were stimulated for 5 days in 24-well plates (106/well) with plate-bound anti-CD3 Ab (1 μg/ml) and soluble anti-CD28 Ab (1 μg/ml) in the presence or not of TGF-β (0.5 or 10 ng/ml) and/or IL-15 (10 ng/ml) and/or IL-2 (300 UI/ml). The suppressive functions of Tregs were tested in co-culture experiments in 96-well plates coated with 0.5 μg/ml UCHT1 (Life Technologies). $CD4^+CD25^-$, $CD3^+$, $CD8^+$, or naive and memory T-subsets (105/well) were plated and added with 1 or 0.25 x 105/well of autologous induced or natural $CD4^+CD25^+$ T-cells in a final volume of 200 μl for 4 to 5 days. For proliferation studies, uptake of [3H]thymidine (Amersham Biosciencs) was measured 18 h after adding 0.4 μCi/well. Cells were harvested and radioactivity was counted in a scintillation counter. For analysis of apoptosis, cells were stained with FITC-annexin V and propidium iodide (TACS Annexin V-FITC; R&D Systems) according to the manufacturer's instructions and the percentage of apoptotic cells positive both for Annexin V and propidium iodide was determined by flow cytometry. To analyze apoptosis of effector cells during co-culture with Tregs, responder cells were stained before culture with CFSE (Molecular Probes) at a concentration of 0.5 μM. After 4 days, cells were harvested, labeled with PE-Annexin V (BD Biosciences) according to the manufacturer's protocol and the percentage of apoptotic Annexin V+ cells was determined by flow cytometry.

IFN-γ detection assay

For IFN-γ detection, capture ELISA was performed on supernatants of cells using Human IFN-γ ELISA Set (BD Biosciences) according to manufacturer's instructions. The limit of detection was 23 pg/ml.

Flow cytometry analysis

Cells (2 x 105) were incubated with FITC, PE, or PE-Cy5.5 conjugated mAbs to human CD3, CD4, CD8, CD25, CD45RA, and CD45RO Abs for 20 min at 4°C. For intracellular FOXP3 detection, cells stained with FITC-conjugated mAb to CD25 were fixed, permeabilized using eBioscience FOXP3 Staining Buffer Set (eBioscience) or Human FOXP3 Buffer Set (BD Biosciences), and labeled with PE-conjugated anti-human Foxp3 (Biosciences or BD Biosciences) or control isotype (BD Biosciences). For intracellular phospho-Akt detection, cells were fixed and permeabilized using Fix Buffer I and Perm Buffer III and labeled with PE-conjugated anti-human phospho-Akt or control isotype (BD Biosciences). For intracellular IFN-γ detection, cells were treated with GolgiStop for the last 6 h of culture, fixed, and permeabilized using BD Cytoperm/Cytofix plus kit and labeled with PE-conjugated anti-human IFN-γ or control isotype (BD Biosciences). Analyses were performed with a FACS Vantage flow cytometer using the CellQuest software (BD Biosciences).

Western blot analysis

Whole-cell or nuclear extracts were obtained from stimulated $CD3^+$ T-cells using the Nuclear Extract kit (Active Motif) according to manufacturer's instructions. Proteins (20 μg) were separated on an 8% sodium-dodecyl-sulfate (SDS-PAGE) gel, transferred to nitrocellulose membranes, and labeled with mAbs against human NFATc2 (Santa Cruz Biotechnology) or human β-actin followed by HRP-conjugated anti-mouse Ab (Cell Signaling Technology). Visualization was performed using ECL system (ECL plus, Amersham Biosciences).

Statistical analysis

Values obtained in the different experiments were compared by the nonparametric Mann-Whitney U test. Statistical significance was assigned to a value of $p < 0.05$.

2.1.3 Results

IL-15 does not impair de novo generation of $CD4^+CD25^+$ Tregs but acts synergistically with TGF-β to convert naive T-lymphocytes into regulatory cells In vitro generation of $CD4^+CD25^+$ Tregs was studied after a 5-day in vitro stimulation of purified $CD4^+CD25^-$ peripheral T-lymphocytes with anti-CD3 and anti-CD28 Abs in the presence or not of TGF-β and/or IL-15. The regulatory function of the generated cells was then evaluated by testing their capacity to inhibit the proliferative response of autologous $CD4^+CD25^-$ T-cells to immobilized anti-CD3 Ab. Stimulation of purified $CD4^+CD25^-$ peripheral T-lymphocytes with anti-CD3 Abs induced the appearance of a very high proportion of cells expressing FOXP3 mRNA and protein but these cells did not exert any suppressive effects (Fig. 1A and data not shown), a finding consistent with the observation that FOXP3 expression can merely reflect

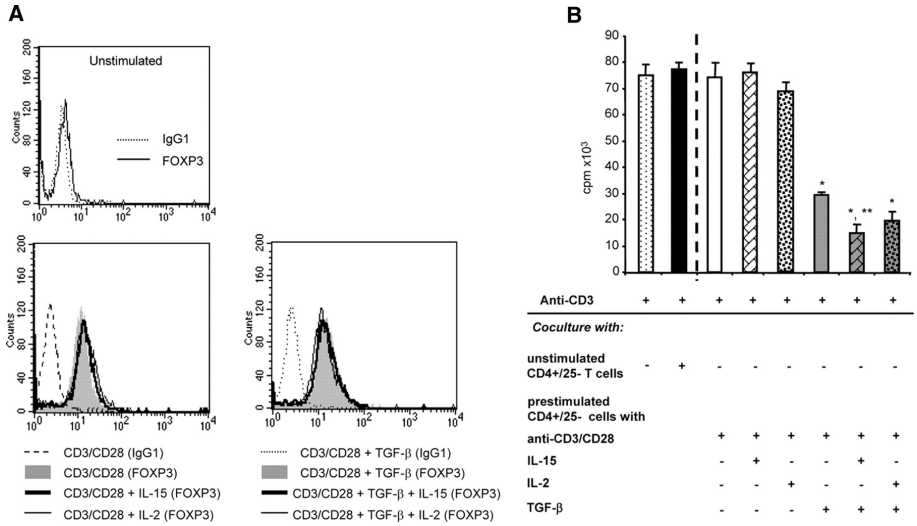

Figure 1. IL-15 does not impair in vitro conversion of conventional T-lymphocytes into Tregs. Purified $CD4^+CD25^-$ peripheral T-lymphocytes were stimulated with immobilized anti-CD3 Ab (1 μg/ml) and soluble anti-CD28 Ab (1 μg/ml) in the presence or not of TGF-β (10 ng/ml) and/or IL-15 (10 ng/ml) and/or IL-2 (300 UI/ml). (**A**) Intracytoplasmic FOXP3 was analyzed by flow cytometry. The analysis was performed on the lymphocyte gate. Results from one representative of three experiments are shown. (**B**), The suppressive function of the generated cells was evaluated by testing their capacity to inhibit the proliferative response of autologous $CD4^+CD25^-$ T-cells stimulated with immobilized anti-CD3 Ab (0.5 μg/ml) at a ratio 1:1 of regulatory to responsive T-cells. Results are from three independent experiments. *, $p < 0.05$ when compared with co-culture with unstimulated $CD4^+CD25^-$ cells. **, $p < 0.05$ when compared with co-culture with $CD4^+CD25^-$ cells stimulated with TGF-β.

T-cell activation in humans (23, 24). Adding TGF-β during the 5-day stimulation had no significant effect on the level of expression of FOXP3-protein or percentage of $CD25^+FOXP3^+$ T-cells (Fig. 1A). In contrast, the latter cells now demonstrated a significant inhibitory effect on the proliferative response of $CD4^-CD25^-$ autologous T-cells analyzed at day 5 (Fig. 1B). Comparable results were obtained when thymidine uptake was measured at earlier time points (day 3) (data not shown). Adding IL-15 had no significant effect on FOXP3-protein expression compared with cultures containing TGF-β alone (Fig. 1A). Yet, $CD25^+FOXP3^+$ T-cells generated in the presence of both IL-15 and TGF-β exerted a significantly stronger inhibitory effect on the proliferation of $CD4^+CD25^-$ T-cells than Tregs generated in the presence of TGF-β alone (Fig. 1B). Comparable results were obtained when IL-15 was replaced by IL-2, but exogenous IL-2 did not enhance significantly the effect of TGF-β on the acquisition of regulatory functions (Fig. 1).

IL-15 impairs the suppressive capacities of natural $CD4^+CD25^+$ Tregs

To test the impact of IL-15 on the suppressive capacities of natural $CD4^+CD25^+$ Tregs, the latter cells were isolated from peripheral blood of healthy donors and their suppressive effects on the proliferation and secretion of IFN-γ were tested on autologous $CD4^+$ or $CD8^+$ T-cells stimulated via their TCR, in the presence or absence of IL-15. As shown in Fig. 2A, adding $CD4^+CD25^+$ T-cells to $CD8^+$ or $CD4^+CD25^-$ purified T-cells at a ratio of 1:1 regulatory to responsive T-cells inhibited their proliferative response to immobilized anti-CD3 by 50%. $CD4^+CD25^+$ cells had an even more striking effect on the production of IFN-γ, which was abolished in $CD8^+$ autologous T-cells at both ratios of 1:1 and 1:4 and abolished or reduced by 80% in $CD4^+$ T-cells. In contrast, in the presence of 10 ng/ml IL-15, $CD4^+CD25^+$ T-cells lost their inhibitory effect on the proliferation and production of IFN-γ (Fig. 2, A and B). Because IL-15 could stimulate the proliferation of $CD4^+CD25^+$ T-cells, the lack of inhibitory effect of the latter cells on T-cell proliferation in the presence of IL-15 may be partly ascribed to the break of their anergic state (Fig. 2A, last columns). Yet, Peluso et al. (25) have recently shown that IL-15 reverted the suppressive effects of $CD4^+CD25^+$ T-cells on the proliferation of CFSE labeled $CD4^+$ and $CD8^+$ lymphocytes. Our results concerning IFN-γ secretion confirm that the effect of IL-15 cannot only be ascribed to a break of anergy of the Tregs. Thus, the inhibitory effect of Tregs on IFN-γ secretion was reverted by IL-15 (Fig. 2B), while IL-15 did not induce any IFN-γ secretion in $CD4^+CD25^+$ T-cells stimulated or not with anti-CD3 (data not shown). Collectively, these data suggest that IL-15 can directly interfere with the regulatory function of $CD4^+CD25^+$ cells and/or the sensitivity of responder cells to Tregs.

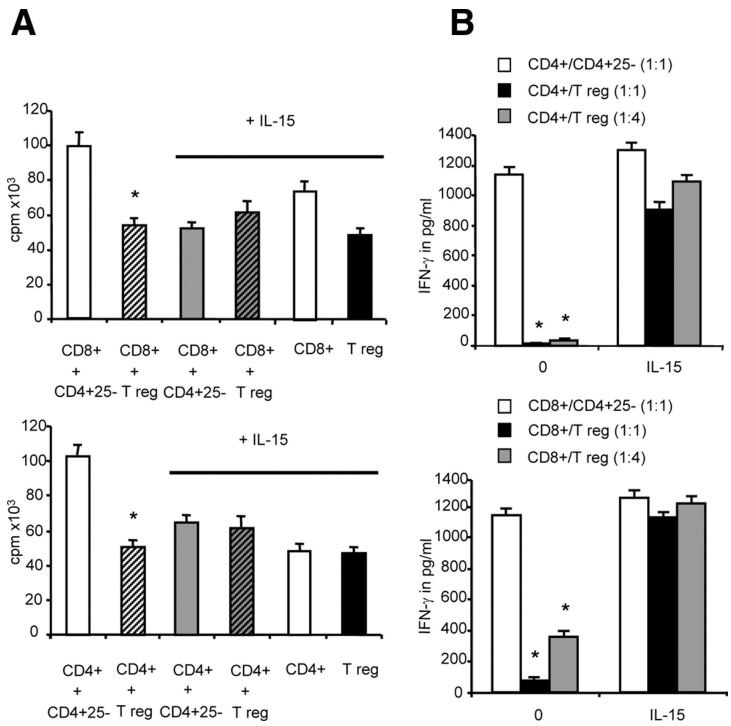

Figure 2. IL-15 impedes the suppressive capacities of natural $CD4^+CD25^+$ Tregs. $CD4^+CD25^+$ Tregs were isolated from peripheral blood of healthy donors and their suppressive effects were tested on autologous $CD4^+CD25^-$ or $CD8^+$ T-cells stimulated with immobilized anti-CD3 in the presence or absence of IL-15. (**A**), Proliferative responses were assessed by [3H]thymidine uptake. Results are from three independent experiments. (**B**), IFN-γ secretion was evaluated in supernatants of co-cultures by an ELISA test. Results are representative of three independent experiments. *, $p < 0.05$ when compared with control co-cultures with $CD4^+CD25^-$ cells.

IL-15 renders conventional T-lymphocytes resistant to the suppressive effects of $CD4^+CD25^+$ Tregs

Consistent with our data indicating that IL-15 does not impair the in vitro generation of $CD4^+CD25^+$ Tregs, several studies have recently shown that IL-15 can expand natural $CD4^+CD25^+$ regulatory T-lymphocytes while preserving their optimal suppressive potency (26, 27). These results suggested that IL-15 did not exert its inhibitory effect by acting directly on Tregs but rather by affecting the sensitivity of responder T-cells to the suppressor effects of Tregs. To test this hypothesis, $CD4^+$ and $CD8^+$ peripheral lymphocytes were incubated or not with IL-15 during 24 h. After extensive washings, the cells were stimulated with anti-CD3 Ab and the inhibitory effects of $CD4^+CD25^+$ Tregs were assessed. Proliferation experiments showed that the suppressive effect of Tregs on peripheral T-cell proliferation was significantly reduced when conventional lymphocytes had been first incubated with IL-15, an

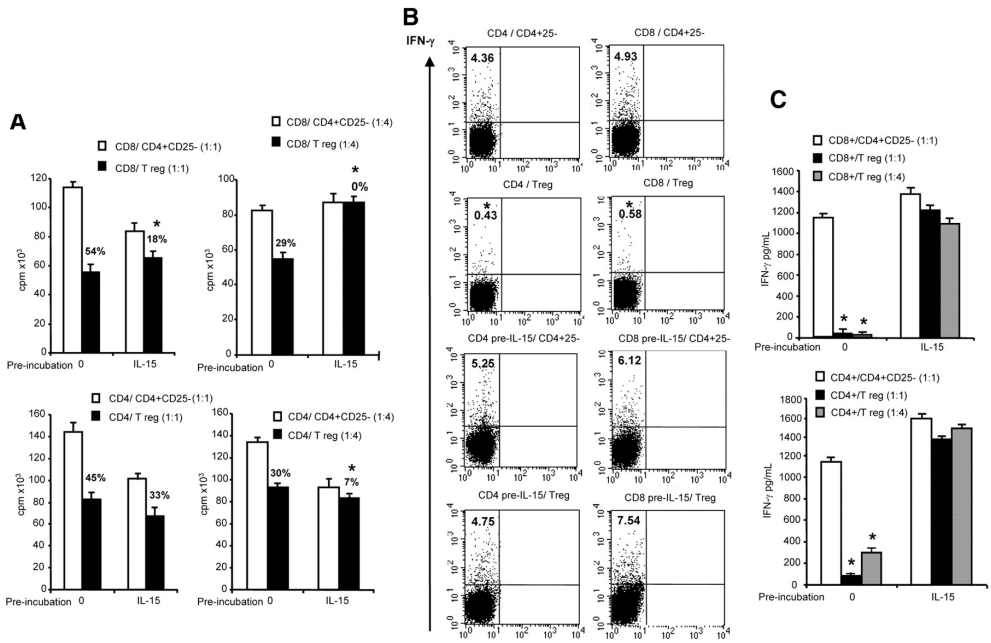

Figure 3. IL-15 renders conventional T-lymphocytes resistant to the suppressive effects of natural $CD4^+CD25^+$ Tregs. $CD4^+$ and $CD8^+$ peripheral lymphocytes were incubated or not with IL-15 (10 ng/ml) during 24 h. Cells were then washed and stimulated with anti-CD3 Ab (0.5 μg/ml) and the inhibitory effects of peripheral $CD4^+CD25^+$ Tregs were assessed on proliferation and IFN-γ production. (**A**), Proliferative responses were measured at day 5 by [3H]thymidine uptake. Results are from six independent experiments. *, $p < 0.05$ when compared with the condition without IL-15 preincubation. (**B**), Intracytoplasmic IFN-γ staining was analyzed by flow cytometry in the lymphocyte gate. Results from one of three independent experiments are shown. Numbers indicate the percentages of positive IFN-γ cells. *, $p < 0.05$ when compared with control co-cultures with $CD4^+CD25^-$ cells. (**C**), IFN-γ secretion was evaluated in supernatants of co-cultures by an ELISA test. Results are from three independent experiments. *, $p < 0.05$ when compared with control co-cultures with $CD4^+CD25^-$ cells.

effect more particularly obvious in the $CD8^+$ subset (Fig. 3A). In addition, preincubation with IL-15 prevented very efficiently the suppressive effect of Tregs on IFN-γ secretion in both $CD4^+$ and $CD8^+$ T-lymphocytes (Fig. 3, B and C). At a 1:1 ratio, the inhibitory effect of Tregs cells on IFN-γ secretion, assessed either by intracytoplasmic staining (Fig. 3B) or by ELISA (Fig. 3C) in the culture supernatants was reduced drastically when lymphocytes had been previously incubated with IL-15 ($p < 0.001$). Altogether, these data demonstrate that IL-15 renders conventional $CD4^+$ and $CD8^+$ T-lymphocytes resistant to the suppressive effects of $CD4^+CD25^+$ Tregs.

IL-15-induced resistance of conventional T-lymphocytes to the suppressive effects of Tregs depends on the PI3K pathway

Recent data indicated that conventional T-cells from NFATc2–/– mice are unresponsive to $CD4^+CD25^+$ Tregs suppression (28). We hence tested whether IL-15 acts on responder cells by modulating NFATc2 pathway. IL-15 had no effect either on NFATc2 protein expression or on NFATc2 induced translocation (Fig. 4A). The mechanism implicated in IL-15 induced resistance of responder cells to suppression was therefore addressed using inhibitors of the major signaling pathways activated by IL-15. As shown in Fig. 4B, only inhibitors of the PI3K/Akt pathway (Wortmaninn and Ly294002), added during the 24-h preincubation of responder cells with IL-15, restored the capacity of the latter cells to respond to the inhibitory effect of Tregs on both T-cell proliferation and IFN-γ secretion. Notably, in the absence of Tregs, responder cells preincubated with IL-15 and PI3K inhibitors exhibited a proliferative response and IFN-γ secretion comparable to that of control responder cells, eliminating a direct effect of these inhibitors on their effector functions (data not shown). Collectively, these data indicate that activation of the PI3K pathway by IL-15 renders T-lymphocytes resistant to the inhibitory effects of Tregs.

The PI3K can also be activated by the related cytokine IL-2 which shares the β and γ-chains of its receptor with IL-15 (29). Yet, the latter chains and the private γ-chains of IL-2 and IL-15 receptors are differentially expressed on T-cell subsets (30). The effects of IL-15 and IL-2 were therefore compared on $CD4^+$ and $CD8^+$ naive and memory T-cell subsets. IL-15 rendered both $CD8^+$ T-cell subsets resistant to the inhibitory effects of Tregs and had a minor but significant effect on $CD4^+$ subsets. In contrast, IL-2 acted preferentially on memory T-lymphocytes, notably $CD4^+$. Comparable results were obtained when analyzing proliferation (Fig. 5A) and IFN-γ secretion (Fig. 5B) by responder cells. Notably, the differential effects of IL-2 and IL-15 in the various T-cell subsets corroborated their capacity to induce PI3K activation as demonstrated by flow cytometry analysis of Akt phosphorylation (Fig. 5C).

Reigniting an old debate, Pandiyan et al. (31) have recently suggested that $CD4^+CD25^+$ Tregs exert their suppressive effects by depriving effector T-cells of life-supporting IL-2, thereby inducing apoptosis of effector T-cells. These authors further showed that the effect of IL-2 involved the PI3K/Akt pathway and that IL-2 could be replaced by other γc cytokines, including IL-15. We were, however, unable to demonstrate that suppression of effector cells was associated with their significant apoptosis (Fig. 6). Thus, no increase in the total percentage of apoptotic cells stained by propidium iodide and annexin V was observed in co-cultures of responder and Tregs compared with responder cells stimulated alone (Fig. 6A), despite the strong inhibitory effect of Tregs on T-cell proliferation (Fig. 6, B and D). To further confirm the lack of Tregs-induced apoptosis of responder cells, the latter cells were labeled before culture with CFSE. CFSE staining decreased in a large fraction of responder cells stimulated alone but not in the presence of Tregs, confirming their suppressive effect

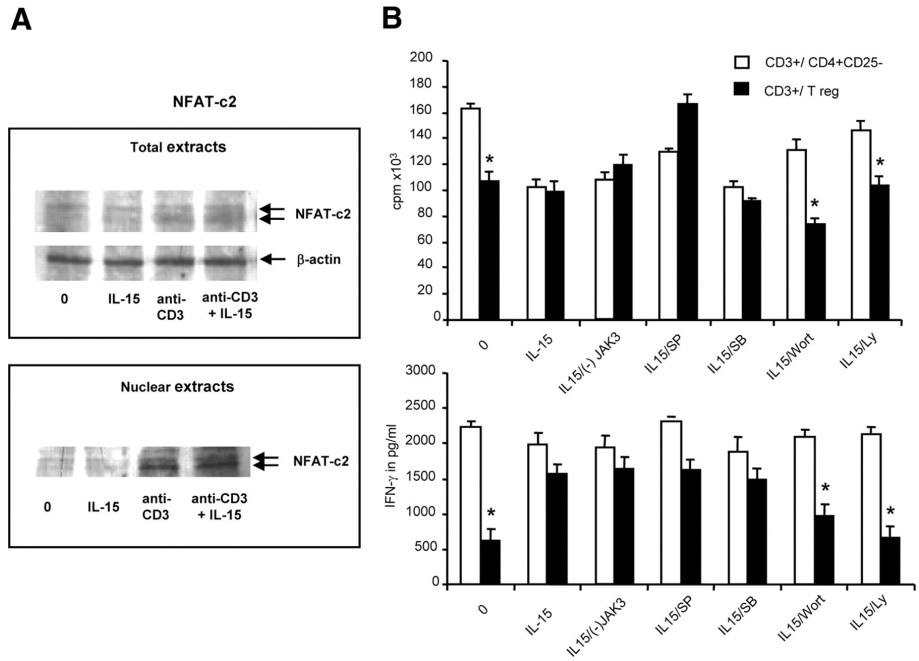

Figure 4. IL-15 acts on conventional T-lymphocytes through activation of PI3K pathway. (**A**), Peripheral $CD3^+$ T-lymphocytes were incubated or not with anti-CD3 (5 μg/ml) in the presence or absence of IL-15 (10 ng/ml) during 24 h. Expression of NFATc2 was evaluated by immunoblotting on total cell extracts (upper panel) and nuclear translocation of NFATc2 was assessed on nuclear extracts (lower panel). (**B**), $CD3^+$ peripheral lymphocytes were incubated or not with IL-15 (10 ng/ml) in the presence JAK3 inhibitor ((-) JAK3), SP600125 (SP), SB203580 (SB), Wortmaninn (Wort), or Ly294002 (Ly) during 24 h. The cells were then washed and stimulated with anti-CD3 Ab and the inhibitory effects of $CD4^+CD25^+$ Tregs on proliferation (upper panel) and IFN-γ secretion (lower panel)

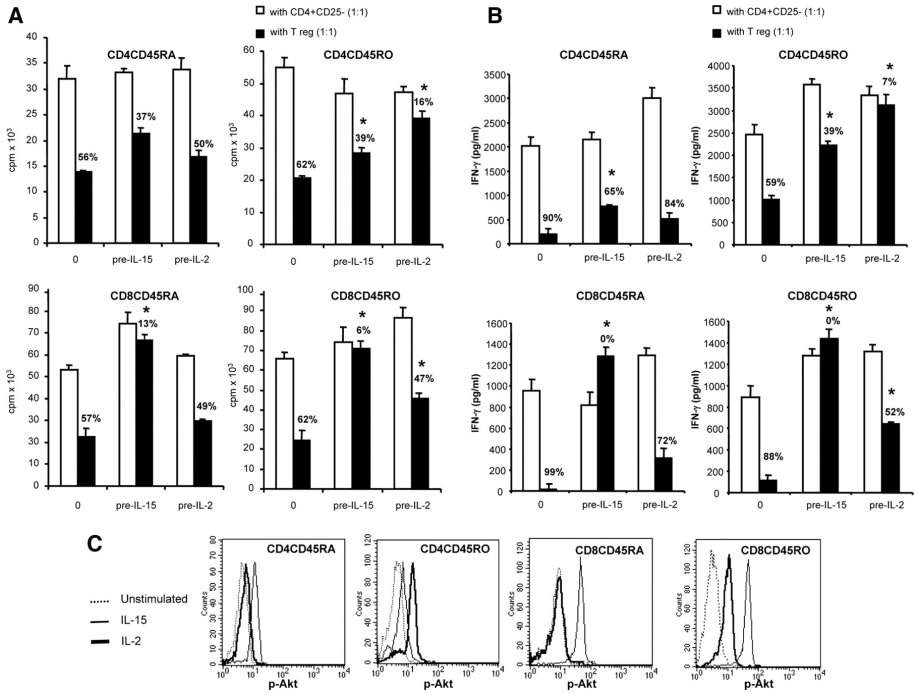

Figure 5. IL-15 acts preferentially on T $CD8^+$ lymphocytes. CD4CD45RA, CD4CD45RO, CD8CD45RA, and CD8CD45RO lymphocytes were incubated or not with IL-15 (10 ng/ml) or IL-2 (300 IU/ml) during 24 h. (**A**), Cells were then washed and stimulated with anti-CD3 Ab (0.5 μg/ml) and the inhibitory effects of peripheral $CD4^+CD25^+$ Tregs were tested at ratio 1:1. Proliferative responses were assessed at day 4 by [3H]thymidine uptake. Results are from three independent experiments. *, $p < 0.05$ when compared with the condition without IL-15 preincubation. (**B**), IFN-γ secretion was evaluated in supernatants of co-cultures by an ELISA test. Results are from three independent experiments. *, $p < 0.05$ when compared with control co-cultures with $CD4^+CD25^-$ cells. (**C**), Intracytoplasmic staining of p-Akt was monitored by flow cytometry. Results from one of three independent experiments are shown.

(Fig. 6B). The percentage of Annexin V+ CFSE+ cells however did not increase in the presence of Tregs (Fig. 6C). Furthermore, inhibitors of PI3K failed to induce any significant apoptosis in $CD4^+CD25^-$ responsive cells stimulated by anti-CD3 and anti-CD28 Abs (Fig. 6A) while they exerted a strong inhibitory effect on their proliferation (Fig. 6D).

2.1.4 Discussion

Our data point out to the ambiguous role of IL-15 in the control of Treg functions and demonstrate that IL-15 promotes the effect of TGF-β on the de novo generation of Tregs but also renders peripheral CD4 and CD8 T-cells resistant to the suppressive functions of Tregs through activation of the PI3K signaling pathway. FOXP3 expression was induced in human $CD4^+CD25^-$ T-cells stimulated by anti-CD3 and anti-CD28 Abs which did not exhibit any Treg functions, confirming previous observations that, in humans, FOXP3 is not a reliable marker of human suppressor cell activity and can simply reflect T-cell activation. In keeping with the presence of STAT5 binding sites in the promoter and in one enhancer of the *FOXP3*-gene (32), recent data have shown that induction of FOXP3 in stimulated human T-cells depends on the activation of STAT5 by the γc cytokines IL-2, IL-7, or IL-15 (33). Yet, STAT5-driven induction of FOXP3 was not sufficient to acquire regulatory properties. This result is coherent with our data showing the lack of suppression by $CD4^+CD25^-$ T-cells activated by anti-CD3/CD28 added or not with IL-15 or IL-2. By contrast, adding TGF-β during the stimulation of $CD4^+CD25^-$ T-cells by anti-CD3 and anti-CD28 Abs resulted in the induction of regulatory properties. In mice, the presence of exogenous TGF-β is necessary during the stimulation by anti-CD3/CD28 Abs to induce both FOXP3 and regulatory functions, and TGF-β plays a non-redundant role with IL-2 in the induction of FOXP3 (11, 34, 35). In humans, adding exogenous TGF-β may not be necessary as Tran et al. observed that 30% of naive $CD4^+$ T-cells expressed FOXP3 in response to TCR stimulation alone. Yet, induction of FOXP3 was prevented by neutralizing TGF-β, and adding TGF-β further increased the proportion of $FOXP3^+$ cells to 80%, confirming the role of this cytokine (35, 36). In our study, FOXP3 mRNA levels increased only very modestly in the presence of exogenous TGF-β and there was no significant increase in level of expression of FOXP3 or proportion of $FOXP3^+$ cells in $CD4^+$ T-cells as compared with cells only stimulated by anti-CD3/CD28 Abs. The impact of exogenous TGF-β may depend on the amounts of TGF-β produced in response to TCR stimulation and/or on the different concentrations of TGF-β present in the serum used for the assay (35). In mice, TGF-β was suggested to stimulate FOXP3 transcription through Smad3 binding to an inverted repeat sequence situated in a 120-bp enhancer fragment on the murine *FOXP3*-gene (37). This result may appear paradoxical given the synergistic effect of IL-15 and TGF-β on the de novo generation of human Tregs observed in the present study and our previous data indicating that IL-15 inhibits Smad3-binding to the promoters of TGF-β target genes in human T-cells (3). Yet, Von Boehmer and Nolting (32) have stressed the role

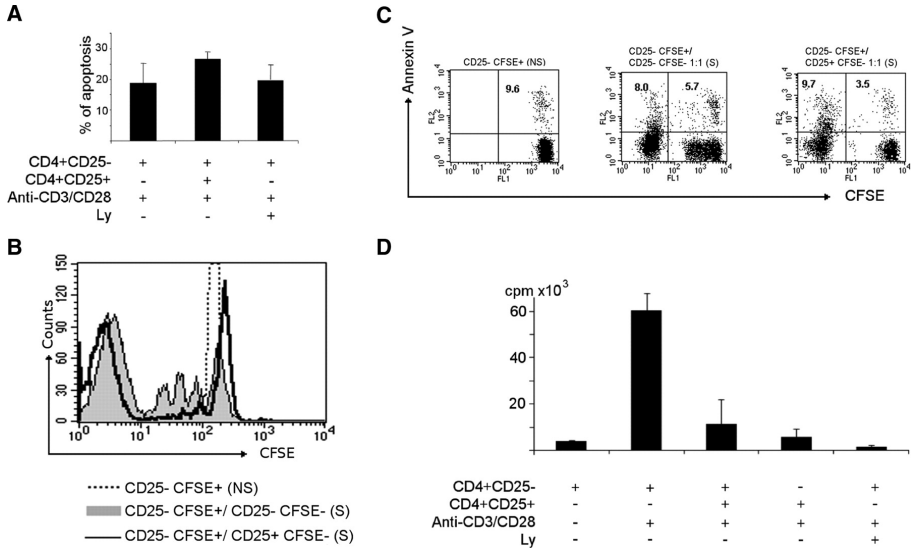

Figure 6. Tregs do not induce apoptosis of responder cells. (**A**), $CD4^+CD25^-$ cells were stimulated by immobilized (0.5–1 μg/ml) anti-CD3 Ab and soluble (1 μg/ml) anti-CD28 Ab in the presence or not of $CD4^+CD25^+$ cells or in the presence of the PI3K inhibitor Ly294002 (Ly) and the percentage of apoptotic cells positive for both annexin V and propidium iodide was assessed after a 5-day culture. Data are representative of three distinct experiments. (**B**), $CD4^+CD25^-$ T-cells were labeled with CSFE and left either not stimulated (NS, dotted line) or stimulated with immobilized anti-CD3 (0.5 μg/ml) and soluble anti-CD28 (1 μg/ml) Abs for 3 days, in the presence of either unlabeled $CD4^+CD25^-$ cells (S, gray-filled histogram) or unlabeled $CD4^+CD25^+$ cells at 1:1 ratio (S, thick full line). The same result was obtained with soluble anti-CD3 (0.75 μg/ml) and anti-CD28 (4 μg/ml) Abs. (**C**), $CD4^+CD25^-$ T-cells were labeled with CSFE and left either not stimulated (NS, left panel) or stimulated with soluble anti-CD3 (0.75 μg/ml) and anti-CD28 (4 μg/ml) Abs for 3 days, in the presence of either unlabeled $CD4^+CD25^+$ cells (middle panel) or unlabeled $CD4^+CD25^+$ cells at 1:1 ratio (right panel). Annexin V and CFSE staining were compared after a 3-day culture in a gate including all cells. Numbers in quadrants are the percentages of annexin V positive cells. Similar results were obtained using immobilized anti-CD3 (0.5–1 μg/ml) and soluble anti-CD28 (1 μg/ml). (**D**), Proliferation of $CD4^+CD25^-$ responder cells stimulated by immobilized anti-CD3 (0.5–1 μg/ml) and soluble anti-CD28 (1 μg/ml) Abs in the presence or not of Ly and cocultured or not with $CD4^+CD25^+$ cells was assessed by [3H]thymidine uptake in the same experiment. Data are representative of four distinct experiments.

of additional Smad3-independent mechanism(s) in TGF-β-dependent induction of FOXP3 transcription in murine T-cells. Furthermore, our results suggest that TGF-β promotes the generation of human Tregs independently of FOXP3 induction. Altogether, these results point out to the likely role of Smad3-independent effect(s) of TGF-β in the generation of Tregs, notably in humans. Contrasting with its promoting effect on the in vitro generation of Tregs, IL-15 hampered the capacity of conventional $CD4^+$ or $CD8^+$ T-cells to respond to the immunosuppressive effects of natural Tregs. IL-15 acted preferentially on $CD8^+$ T-cells either naive or memory while the related cytokine IL-2 had a preferential effect on memory lymphocytes, particularly $CD4^+$. These results are in keeping with the known distribution of IL2 and IL-15 receptor chains on T-cell subsets (30). We found no evidence that IL-15 may modulate NFATc2 recently involved in the capacity of murine effector cells to respond to Tregs (28). In contrast, our results point to the central role of the PI3K pathway. Phospho-Akt was induced after IL-15 stimulation, particularly in $CD8^+$ T-lymphocytes and inhibition of PI3K pathway impeded IL-15 effects on responder cells. The key role of PI3K pathway in inducing resistance to suppressive effects of Tregs is in keeping with the recent hypothesis put forward by Wolfert et al. (38). Based on the observation that several mouse models of autoimmunity either spontaneous (NOD, MLR/Mp, etc.) or in engineered mice (Cbl-b–/–, TRAF6–/– etc.) show simultaneous resistance of effector T-cells to Tregs and hyperactivity of the PI3K/Akt pathway (38), these authors postulated that the activation status of the PI3K/Akt pathway in conventional T-cells is a primary determinant of cell sensitivity to Treg-mediated suppression. The role of PI3K/Akt pathway in regulating Treg-mediated suppression remains, however, unclear. Considering the recent data obtained by Pandiyan et al. (31), we tested whether $CD4^+CD25^+$ Tregs exert their suppressive effects by inducing apoptosis of effector T-cells and whether IL-15, alike other γc cytokines may impair this effect by activating PI3K/Akt pathway. We were, however, unable to demonstrate that suppression of effector cells was associated with their significant apoptosis. Furthermore, inhibitors of PI3K pathway failed to induce any significant apoptosis in stimulated $CD4^+CD25^-$ responsive cells. It is unclear why our data in humans differ from recent data in mice. Yet, our results are consistent with previous studies performed either on mice or humans. Accordingly, Duthoit et al. (39) observed that stimulated $CD4^+CD25^-$ T-cells failed to up-regulate IL-2 mRNA in the presence of Tregs, and that exogenous IL-2 did not break the mitotic blockage of stimulated $CD4^+CD25^-$ T-cells cocultured with Tregs while promoting their survival. Furthermore, Fontenot et al. (40) showed that $Foxp3^+CD4^+$ T-cells from IL2ra–/– mice possess equivalent regulatory functions as their counterparts in wild-type mice. Finally, Oberle et al. (41) demonstrated that in humans, suppression of IL-2 and IFN-γ mRNA by Tregs was not dependent on IL-2 consumption. Besides an effect based on sopping IL-2, a plethora of mechanisms have been evoked in mediating immunosuppression by Tregs (42, 43). Future work will be necessary to decipher which of these mechanisms implicate(s) the PI3K/Akt

pathway. Finally, it may be stressed that our study analyzes how IL-15 interferes with the direct effect of Tregs on effector T-cells. Recent studies indicate that Tregs can exert their suppressor effect by inhibiting dendritic cell maturation and Ag-presentation (44). Because IL-15 is a potent inducer of both functions (45), it will be interesting to assess whether IL-15 might also prevent this effect of Tregs. In conclusion, our in vitro data point to the ambiguous role of IL-15 in the control of Treg functions. On the one hand, IL-15 can promote the generation of induced FOXP3$^+$ Tregs. In contrast, IL-15 can render effector cells either CD4$^+$ or CD8$^+$ unresponsive to the regulatory effect of FOXP3$^+$ Tregs. The latter effect of IL-15 is reminiscent of IL-6. Thus, IL-6, released by TLR-activated dendritic cells, rendered responder T-cells refractory to the suppressive effect of Tregs and this effect was suggested to promote a rapid and efficient immune response against pathogens (46). IL-15 is one cytokine rapidly but transiently induced in response to intracellular pathogen (47). The dual effect of IL-15 demonstrated in the present study may be beneficial to promote a rapid inflammatory response to pathogens and simultaneously prepare its subsequent down-regulation after elimination of the pathogen. Conversely, the impact of IL-15 on responding T-cells may become deleterious in situations associated with protracted IL-15 over-expression, preventing the return to homeostasis and sustaining inflammation. Over-expression of IL-15 has been observed across a range of pro-inflammatory and autoimmune-related human diseases including psoriasis, rheumatoid arthritis, and celiac disease (7, 48). Our data indicating that IL-15 counteracts the suppressive action of Tregs point to their probable dysfunction in diseases associated with IL-15 disordered over-expression. This effect of IL-15 together with its inhibitory effect on Smad3 signaling, provides further insight on how protracted IL-15 production may contribute to the loss of immune tolerance and further support therapeutic strategy targeting IL-15 in diseases associated with IL-15 deregulation.

2.1.5 References

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CHAPTER 3

HETEROGENEITY IN AIE

Severe Food Allergy as a Variant of IPEX Syndrome Caused by a Deletion in a Non-coding Region of the FOXP3-Gene.

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Reduced expression of FOXP3 and regulatory T-cell function in severe forms of early-onset autoimmune enteropathy.

Moes N, Rieux-Laucat F, Begue B, Verdier J, Neven B, Patey N, Torgerson TT, Picard C, Stolzenberg MC, Ruemmele C, Rings EH, Casanova JL, Piloquet H, Biver A, Breton A, Ochs HD, Hermine O, Fischer A, Goulet O, Cerf-Bensussan N, Ruemmele FM.

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3.1 Severe Food Allergy as a Variant of IPEX Syndrome Caused by a Deletion in a Non-coding Region of the *FOXP3*-Gene

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Gastroenterology. 2007 May;132(5):1705-17.

Abstract

BACKGROUND and AIMS:

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX; OMIM 304930) syndrome is a congenital syndrome characterized by autoimmune enteropathy, endocrinopathy, dermatitis, and other autoimmune phenomena. In the present work, we aimed to uncover the molecular basis of a distinct form of IPEX syndrome presenting at the edge of autoimmunity and severe allergy.

METHODS:

The FOXP3-gene was sequenced, FOXP3-messenger-RNA (mRNA) was quantified by real-time polymerase chain reaction (PCR), and protein expression in peripheral blood lymphocytes was analyzed by flow cytometry after intracellular staining. In coculture experiments ($CD4^+CD25^-$ and $CD4^+CD25^+$ cells), the functions of regulatory T-cells were analyzed. Expression of interferon gamma and interleukin 2 and 4 mRNA within the inflamed intestinal mucosa was quantified by real-time PCR.

RESULTS:

Here, we describe a distinct familial form of IPEX syndrome that combines autoimmune and allergic manifestations including severe enteropathy, food allergies, atopic dermatitis, hyper-IgE, and eosinophilia. We have identified a 1388-base pair deletion (g.del-6247_-4859) of the FOXP3-gene encompassing a portion of an upstream noncoding exon (exon -1) and the adjacent intron (intron -1). This deletion impairs mRNA splicing, resulting in accumulation of unspliced pre-mRNA and alternatively spliced mRNA. This causes low FOXP3-mRNA levels and markedly decreased protein expression in peripheral blood lymphocytes of affected patients. Numbers of $CD4^+CD25^+FOXP3^+$ regulatory T-cells are extremely low, and the $CD4^+CD25^+$ T-cells that are present exhibit little regulatory function.

CONCLUSIONS:
A new mutation within an upstream noncoding region of FOXP3 results in a variant of IPEX syndrome associating autoimmune and severe immunoallergic symptoms.

3.1.1 Introduction

An old but initially speculative immunologic concept claiming the existence of suppressor T-lymphocytes (1,2) was substantiated with the discovery that mutations of the transcription factor FOXP3 result in the immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (3,4,5) and that FOXP3 is required for the generation of $CD4^{+}CD25^{+}$ regulatory T-lymphocytes (Treg). (6,7,8) A growing body of experimental and in vivo evidence in humans and animals has demonstrated a crucial role for FOXP3-expressing $CD4^{+}$ T-cells as potent suppressors of self-reactive T-cell activation and proliferation, presumably via direct cell-cell interaction. (9,10) Some evidence points to a role for Treg cells in the control of immune responses to exogenous antigens, such as dietary food antigens, (11,12) but much less is known about this aspect of their function.
The *FOXP3*-gene maps to Xp11.23 and encodes a 431-amino acid (48 kilodalton) protein, also named scurfin. FOXP3 has significant homology to members of the Forkhead/winged-helix transcription factor family (13) and plays a central role in the generation of Treg cells. FOXP3 functions as transcriptional repressor, thus allowing negative control of T-cell activation via DNA sequences containing FOXP3 binding sites located adjacent to nuclear factor of activated T-cell regulatory sites in cytokine promoters such as interleukin (IL)-2 or granulocyte-macrophage colony-stimulating factor enhancer. (14,15,16) IPEX syndrome is a severe, systemic autoimmune disorder that typically presents in infancy with various autoimmune symptoms including protein-loosing enteropathy; early onset, insulin-dependent diabetes mellitus; and other endocrinopathies, dermatitis, and autoimmune cytopenias.(17,18,19,20) Effective therapeutic options for IPEX syndrome patients are limited. In addition to supportive measures, such as total parenteral nutrition, insulin injections, thyroid hormone substitution, and red blood cell transfusions, various immunosuppressive regimens have been utilized including high-dose steroids, cyclosporin A, tacrolimus, sirolimus, and rituximab with variable success.(19,20,21,22,23) Because IPEX syndrome results from an absent or dysfunctional lymphocyte subset, bone marrow transplantation offers a potentially curative treatment option for boys suffering from this devastating disorder. (24,25,26) Results thus far have been mixed, but efforts are underway to optimize conditioning regimens to improve further this mode of therapy. Here, we present a kindred with a distinct clinical presentation of IPEX syndrome with no detectable endocrinopathy but an impressive allergic phenotype manifested by severe food allergy and eczema. Molecular analyses revealed a unique *FOXP3*-mutation involving a large deletion in an upstream, non-coding region of the *FOXP3*-gene. This deletion, encompassing the 3′ half of the untranslated -1 exon and approximately 1000 base pairs (bp)

of the adjacent intron, leads to markedly reduced FOXP3 messenger RNA (mRNA) levels and absence of regulatory T-cells. These cases provide a unique insight into the critical role of naturally arising $CD4^{+}CD25^{+}FOXP3^{+}$ Treg cells in controlling immune responses to exogenous antigens as well as in maintaining self-tolerance.

3.1.2 Clinical Features

Patients IV.1 and IV.2

Patient IV.1, (Figure 1) the index case, followed at Necker-Enfants Malades Hospital, Paris, was born in 2000 after an uneventful pregnancy to unrelated, healthy white parents.

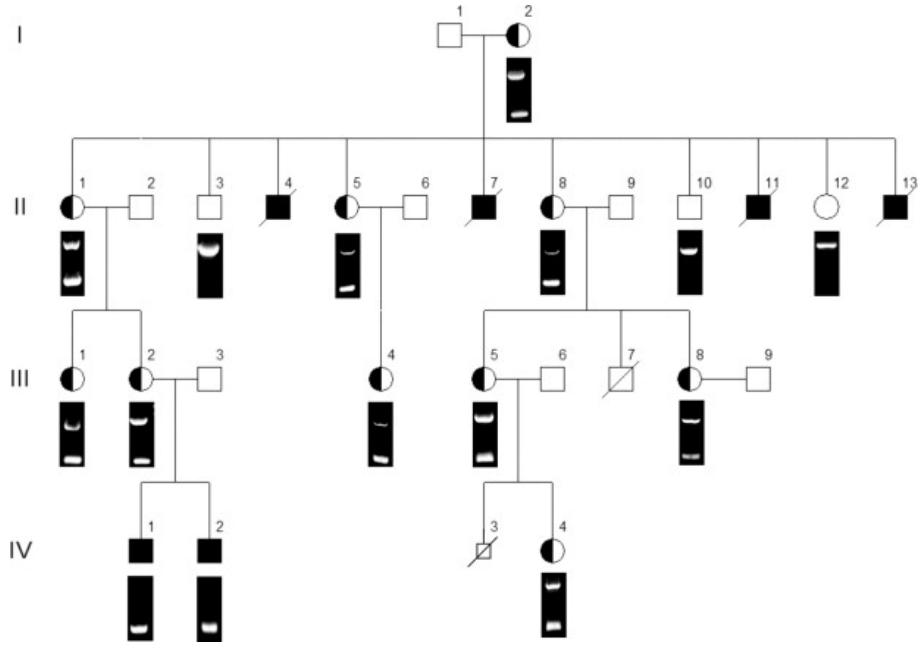

Figure 1. Pedigree demonstrating X-linked inheritance in the identified kindred. Individual family members were tested for the presence of the identified deletion mutation using PCR with forward and reverse primers flanking the deletion in genomic DNA. These primers generate an 1805-base pair fragment from a normal allele and a 417-base pair fragment from an allele harboring the identified 1388-base pair deletion. An ethidium bromide-stained gel demonstrating the fragments amplified from genomic DNA is shown beneath each of the individuals who were tested. Note that the proband (IV.1) and his brother (IV.2) demonstrate only the mutant allele, whereas normal, unaffected males demonstrate only the wild-type allele (II.3 and II.10). All but one of the females tested were found to be carriers of the mutation.

No abnormalities were observed during the neonatal period while he was exclusively breastfed. At 3 weeks of age, infant formula was introduced, and, within 1 week, he developed massive

watery-bloody diarrhoea requiring total parenteral nutrition, daily albumin supplementation, and repeated blood transfusions. The severe protein-loosing enteropathy was accompanied by the appearance of an erythematous, eczematous skin rash. Laboratory evaluation revealed no signs of glucose intolerance, thyroid abnormalities, Addison's disease, or hematologic abnormalities, and there was no evidence of renal or lung involvement. Immunologic testing at the age of 6 weeks showed normal peripheral blood lymphocyte counts and subsets (3800/μL: $CD3^+$, 91%; $CD4^+$, 65%; $CD8^+$, 21%; CD19, 5%) with reduced natural killer (NK) T-cells ($CD3^+CD56^+$, 0.6%) and normal levels of IgG (200 mg/dL), IgM (15 mg/dL), and IgA (26mg/dL). In contrast, IgE levels were strikingly elevated at $>$3000 μ/mL and associated with eosinophilia (950 cells/μL). At the age of 2 months, high titer anti-enterocyte antibodies (immunofluorescence study) were demonstrated. Anti-autoimmune enteropathy (AIE) 75-kilodalton antibodies [27] (directed against intestinal epithelial cell antigens) were initially negative but became strongly positive within the first 4 months of life. No other auto-antibodies were detected, including anti-nuclear, anti-liver, anti-kidney, anti-smooth muscle, anti-mitochondrial, anti-microsomal, or anti-thyroglobulin antibodies. Initial endoscopic evaluation of the gastrointestinal (GI) tract, performed at 7 weeks of age, revealed gastric hyperemia and villous atrophy in the duodenum. Histologic analysis showed an intense lympho-plasmocellular infiltrate with a marked eosinophilic component in the lamina propria of the stomach as well as the duodenum (Figure 2).

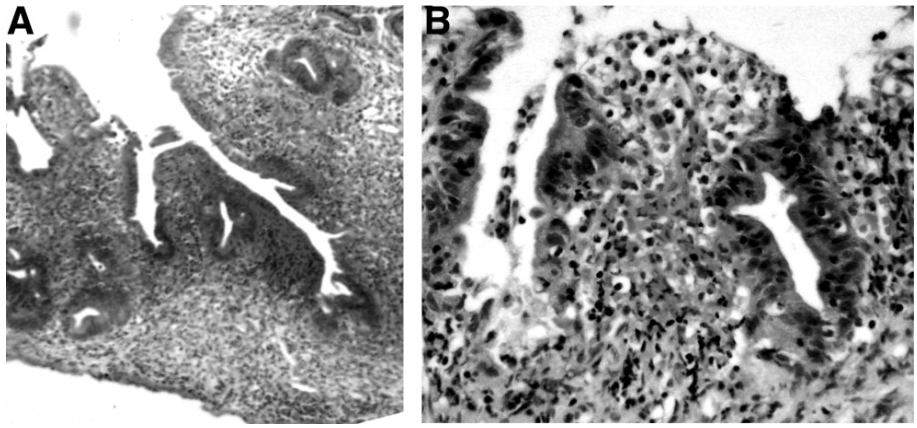

Figure 2. Histologic analysis of a duodenal section of patient IV.1 before treatment. At lower magnification (A; original magnification, 10×), total to subtotal villous atrophy along with a massive mononuclear infiltration of the lamina propria and severe epithelial alterations can be observed. (B) At higher magnification (original magnification, 40×), the infiltrate is predominantly of mononuclear origin with few scattered eosinophilic lymphocytes. A crypt abscess of polynuclear lymphocytes is seen in a crypt, which is almost completely destroyed (dedifferentiated epithelial cells and high degree of apoptotic enterocytes). H&E stain.

The epithelial layer was disorganized with a high rate of apoptosis among the enterocytes. Duodenal biopsy specimens were characterized by severe to total villous atrophy and a massive T-cell infiltration in the lamina propria, with no or only moderate increase of intraepithelial lymphocytes. The colonic mucosa revealed superficial ulcerations and a major mononuclear cell infiltrate that was rich in eosinophils. Neither bacteria nor viral pathogens could be identified in the intestinal/colonic mucosa or in peripheral blood. Patient IV.2, the younger brother of the index case, was born at term in 2002 after an uneventful pregnancy and delivery. Because of the older brother's medical history, the mother's diet was restricted beginning during the third trimester of pregnancy to exclude cow milk protein and other common allergens including eggs, peanuts, and fish. After birth, the patient was exclusively breastfed. Despite these measures, he developed clinical symptoms at 2 months of age, similar to those of his older brother, including eczema and protein-loosing enteropathy with severe watery-bloody diarrhoea requiring total parenteral nutrition, daily albumin supplementation, and red cell transfusions. There were no signs of glucose intolerance, thyroid dysfunction, or other organ involvement. Immunologic testing at the age of 2 months showed normal peripheral blood lymphocyte counts and subsets (3200/μL: $CD3^+$, 90%; $CD4^+$, 64%; $CD8^+$, 20%; $CD19^+$, 6%) with a slightly decreased NK T-cell population ($CD3^+CD56^+$, 2%). IgG, IgM, and IgA levels were normal (300 mg/dL, 38 mg/dL, and 18 mg/dL, respectively). Like the index case, IgE levels were markedly elevated (365 IU/mL at 2 weeks of age) and worsened over time (>2000 IU/mL at 2 months of age) with an accompanying eosinophilia (2400/μL).

At the onset of symptoms, he was positive for antienterocyte antibodies with pronounced staining along the brush border membrane, anti-AIE 75-kilodalton antibodies, and low titer antismooth muscle auto-antibodies. Antinuclear, antithyroglobulin, anti-Langerhans islet, and anti-insulin antibodies were negative. Both boys (patients IV.1 and IV.2) were treated similarly with a triple-immunosuppressive regimen, consisting of 3 steroid pulses combined with tacrolimus (serum levels, 8–10 ng/mL) and azathioprine (1.5 mg/kg). In each case, the clinical response was prompt, with resolution of the protein-loosing enteropathy allowing cessation of parenteral nutrition within 1.5 months. Steroids were tapered and stopped after 3 months. Serum IgE levels decreased but never became normal. All inflammatory parameters (erythrocyte sedimentation rate, C-reactive protein) reverted to normal. After 6 weeks of immunosuppressive treatment (prior to oral alimentation), small and large bowel histology of both patients showed normal cytoarchitecture with a marked reduction in the number of inflammatory cells in the lamina propria. Oral alimentation with an elemental diet (NEOCATE; Nutricia, Gaithersburg, MD) was introduced and tolerated by both. Subsequently, both patients developed multiple skin and food allergies, including erythema, eczema, urticaria, abdominal pain, and watery diarrhoea. Allergic skin reactions worsened when oral diversification was started in the older brother (patient IV.1), despite exclusion of all major allergic antigens, such as cow's milk proteins, eggs, peanuts, exotic fruits, or fish. Identical immediate allergic reactions occurred in both boys after the accidental ingestion of minimal traces of cow's milk protein in cookies. These reactions were characterized by immediate vomiting, followed by abdominal pain for 24 hours and liquid stools for 24 hours. In addition, the eczema worsened markedly. In patient IV.1, several episodes of lip swelling, buccal edema, and vomiting were observed upon ingestion of peanut-contaminated chocolate or other food allergens, which could not be identified. Similar episodes occurred in patient IV.2, although less frequently. IgE against cow's milk proteins was repeatedly documented in both children. No bronchopulmonary symptoms were observed except for an episode of Pneumocystis carinii pneumonia in patient IV.2, 3 weeks after initiation of immunosuppressive therapy. This resolved with trimethoprim sulfamethoxasol treatment. After switching from tacrolimus to sirolimus combined with azathioprine, gastrointestinal manifestations could be controlled and skin symptoms reduced to mild flare-ups triggered by alimentary allergens. Auto-antibodies (against enterocytes and anti-AIE 75 kilodaltons) disappeared and have remained negative. Both children are in remission for almost 6 and 4 years, respectively, but show delayed growth and variable eosinophilia.

Patient III.7

Patient III.7 was the second child of healthy parents, born at term in 1971 without any apparent problems. He died at 2 weeks of age of unknown causes and without any evidence of infection or gastrointestinal manifestation. No autopsy was performed. This patient was

the maternal uncle of patient IV.3; his mother is a carrier of the *FOXP3*-mutation.

Patients II.4, II.7, II.11, II.13

All 4 brothers were born at term between 1946 and 1960 to healthy non-consanguineous parents; the mother is a carrier of the identified *FOXP3*-mutation. After an initial uncomplicated neonatal period, each child developed symptoms of enteropathy, and all died within the first 2 months of life. Patient II.4, initially exclusively breastfed, died at the age of 5 weeks with severe diarrhoea after the introduction of cow's milk. Patient II.7 died at the age of 6 weeks after the onset of intractable diarrhoea while still breastfed. Similar clinical symptoms were reported for patients II.11 and II.13, who died at the age of 3 weeks and 8 weeks in 1954 and 1960, respectively. No information is available regarding whether any of these 4 boys presented with additional symptoms, such as endocrinopathy or skin disease.

Other Family Members

A male fetus (patient IV.3) was miscarried at 6 months of pregnancy with no apparent explanation. No morphologic abnormalities were documented, and no necropsy was performed. The mother is a second cousin of patients IV.1 and IV.2, and she and her daughter (patient IV.4) were found to be carriers of the identified *FOXP3*-mutation. Ten asymptomatic females spanning 4 generations were found to be heterozygous for the same *FOXP3*-mutation as present in patients IV.1 and IV.2 (Figure 1). IgE levels were normal, and none of them reported food or cow's milk protein allergy during infancy. In addition, no female carrier suffered from asthma or atopic skin disease, enteropathy, insulin-dependent diabetes, glucose intolerance, or other endocrinopathies. Using available genomic DNA, a large deletion involving the *FOXP3*-gene was identified in 12 members of this family. The presence of the deletion only in the affected males (patients IV.1 and IV.2) and not in asymptomatic males (patients II.3 and II.10) strongly suggests that it is pathogenic. It is probable that the 4 brothers presenting with GI symptoms who died within the first 2 months after birth (patients II.4, II.7, II.11, II.13) were also carrying this mutation.

3.1.3 Materials and Methods

Isolation of Peripheral Blood Mononuclear Cells Peripheral blood mononuclear cells (PBMNC) were isolated from whole blood using Ficoll-Paque gradient centrifugation as previously described. (5)

Sequence Analysis of FOXP3

Genomic DNA (gDNA) was isolated from whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Each of the 11 coding exons

of FOXP3 including intron/exon boundaries and the region surrounding the first polyadenylation signal were amplified from gDNA by polymerase chain reaction (PCR) using intronic primer pairs as previously reported. (5,28) PCR products were separated by 1% agarose gel electrophoresis, purified with the QIAquick PCR Purification Kit (Qiagen) and directly sequenced using the BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Boston, MA). Rapid genotyping of family members (Figure 1) was accomplished by PCR amplification of fragments from gDNA using primers flanking the identified deletion: forward, 5′-CTCTGTGGTGAGGGGAAGAAATC-3′; reverse, 5′-CATGAATGGCCAATGAATAGTAAAAG-3′. Using these primers, the normal allele yields an amplicon of 1805 bp, and the mutated allele yields an amplicon of 417 bp.

Quantitative Real-Time PCR of FOXP3 mRNA

Total RNA was extracted from 107 fresh PBMNCs using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First strand complementary DNA (cDNA) synthesis was performed using the Omniscript RT Kit (Qiagen) per the manufacturer's protocol. FOXP3 cDNA levels were quantitated using an iCycler iQ Real-Time PCR System (Bio-Rad, Hercules, CA) and Platinum SYBR Green qPCR Supermix-UDG (Invitrogen). Amplification was carried out in a total volume of 20 μL for 40 to 50 cycles with denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. Samples were run in triplicate, and their relative expression was determined by normalizing to expression of the housekeeping gene GAPDH. Values obtained were compared with the level of expression in a normal control sample to calculate the relative fold change. Primers for FOXP3 bind across the spliced junctions of exons 8 and 9 and 9 and 10 distal to the exons affected by the identified mutation: 5′-CTGCCCACACTGCCCCTAGTC-3′ and 5′-CCATTTGCCAGCAGTGGGTAG-3′. GAPDH primers: 5′-CCACATCGCTCAGACACCAT-3′ and
5′-GGCAACAATATCCACTTTACCAGAGT-3′.

Cytokine Profile Within the Intestinal Mucosa

Duodenal biopsy specimens obtained before and 6 weeks after initiation of immunosuppressive therapy were stored in RNAlater (Ambion; Applied Biosystems, Foster City, CA). Total RNA was extracted from the biopsy material using the RNAeasy Mini Kit (Qiagen, Courtaboeuf, France) and reverse transcribed using Murine-Moloney leukemia virus reverse transcriptase, random hexamers, and oligo dT (Promega, Charbonnieres, France) according to standard protocols. In parallel, total RNA was obtained from mucosal biopsy specimens of children with active celiac disease (n = 5), Crohn's disease (CD) (n = 5), or noninflammatory controls (n = 5) as well as 2 additional biopsy specimens of patients IV.1 and IV.2 during follow-up endoscopies. For quantitative real-time PCR, amplifications were performed using Taqman gene assays for interferon (IFN)-γ, IL-2, IL-4, and GAPDH (Applied Biosystems, Paris, France).

Forty cycles of amplification were performed with denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute using an ABI PRISM 7700. For IFN-γ, IL-2, and IL-4, results were normalized to the housekeeping gene GAPDH using the $\Delta\Delta$CT method after controlling that efficiencies of target and reference amplifications were equal or close to 1. (29)

Flow Cytometry of FOXP3$^+$ CD4$^+$CD25$^+$ Cells

Surface staining for CD3, CD4, and CD25 was performed on freshly isolated PBMNCs. Cells were then washed in phosphate-buffered saline (PBS) and fixed for 30 minutes at 4°C using the eBioscience fixation/permeabilization kit, according to manufacturer instructions. After washing (PBS) and permeabilization (15 minutes at 4°C using the eBioscience permeabilization buffer supplemented with 4% normal rat serum), the cells were incubated with anti-human FOXP3 antibody (clone PCH101, eBioscience) overnight at 4°C. After 3 additional wash steps, the labelled cells were analyzed by flow cytometry (BDLaser), gating on CD3$^+$ cells.

Co-culture Experiments of CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T Cells CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T-cells were isolated using a CD4$^+$CD25$^+$ regulatory T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The CD4$^+$CD25$^-$ cells obtained using this approach were approximately 90% pure, and the CD4$^+$CD25$^+$ cells were >95% pure as assessed by flow cytometry. Suppressor activity of the CD4$^+$CD25$^+$ T-cells was evaluated using a standard coculture technique with 50,000 CD4$^+$CD25$^-$ effector cells and 10,000 CD4$^+$CD25$^+$ regulatory T-cells per well in the presence of plate-bound anti-CD3 antibody (UCHT1 1 μg/mL; Beckmann Coulter, France). After 5 days of culture, cells were pulsed with H3-thymidine for 18 hours, and DNA synthesis was estimated as previously described. (30) Suppressor activity of the CD4$^+$CD25$^+$ cells was determined as the difference in the proliferation rate observed when anti-CD3-stimulated CD4$^+$CD25$^-$ T-cells were cultured alone or in the presence of the CD4$^+$CD25$^+$ T-cells. This study was approved by the local ethics committee, and both parents as well as all involved family members gave written consent for these analyses.

3.1.4 Results

Genetic Analysis

The human *FOXP3*-gene consists of 11 protein-coding exons (exons 1–11) as well as a noncoding exon (exon -1) located 6 kilobases (kb) upstream of exon 1 (Figure 3).

All mutations of the *FOXP3*-gene identified to date are located within, or adjacent to, one of the 11 translated exons or within the first polyadenylation site. Based on the clinical phenotype observed in affected members of this family, we sequenced exons 1–11 of the

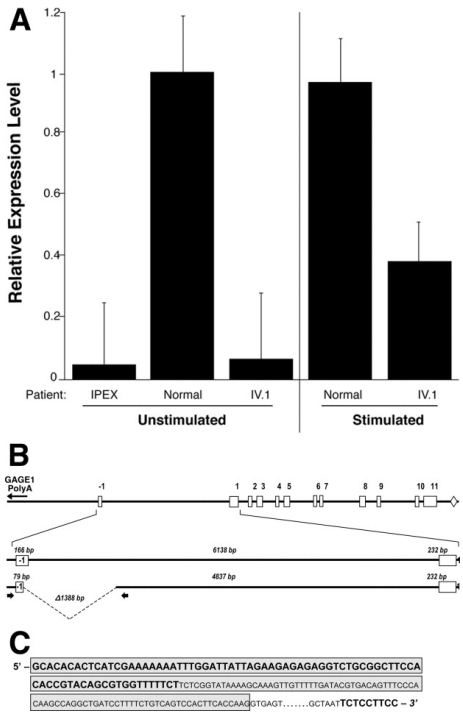

Figure 3. (A) FOXP3 mRNA levels are low in the proband. FOXP3 mRNA levels in fresh or PHA/IL-2 stimulated PBMNCs were quantified using quantitative real-time PCR. Expression levels were normalized to the housekeeping gene GAPDH. Data are shown as relative expression with the control normalized to an expression level of 1.0. Error bars show the standard deviation of 3 independent runs. The expression level in the proband (IV.1) is shown relative to a normal control and a patient with IPEX syndrome who harbors a mutation in the polyadenylation site of the *FOXP3*-gene that leads to mRNA instability.31 (B) Genomic structure of the human FOXP3 locus demonstrating the position of the identified deletion. The position of the protein coding exons (1-11) are shown relative to the single upstream non-coding exon (-1) and the transcriptional start site and direction of the next upstream gene, GAGE1. The region containing exons -1 and 1 with the intervening intron is expanded to show the location of the identified 1388-bp deletion mutation (represented by the dashed line). The arrows flanking the deletion show the locations and directions of the primers used to amplify the fragments that allowed rapid screening of family members (Figure 1). (C) Genomic sequence of the identified deletion mutation. The deleted fragment is shown in small font with the remaining flanking genomic DNA sequence shown in large bold font. The extent of exon -1 is shown within the shaded box demonstrating that the deletion includes the 3′ half of exon -1 including the splice donor site.

FOXP3-gene, including all intron/exon boundaries and the first polyadenylation site in patient IV.1 but found no mutation.

The clear X-linked inheritance pattern in the setting of an IPEX-like phenotype suggested the possibility of a defect in a regulatory region of the FOXP3 genomic locus that might lead to decreased expression of the gene product. To evaluate this possibility, we quantified FOXP3 mRNA levels in PBMNC from patient IV.1 using gene-specific primers and real-time PCR. This patient was found to have expression levels comparable with a known IPEX syndrome patient whose mutation in the first polyadenylation site leads to mRNA instability and rapid mRNA degradation (31) (Figure 3A). Both were significantly low relative to the normal control (5%–7% of normal) (Figure 3A). FOXP3 expression is limited almost exclusively to the $CD4^+FOXP3^+$ regulatory T-cell population in normal PBMNC. Because the $CD4^+FOXP3^+$ regulatory T-cell population is markedly diminished or absent in many patients with IPEX syndrome, (32) low FOXP3 mRNA expression may reflect a paucity of FOXP3-expressing cells and not actually low mRNA expression on a per cell basis. To address this question, FOXP3 mRNA was also quantified in PBMNC stimulated with phytohemagglutinin (PHA) (10 μg/mL) and IL-2 (100 U/mL). We and others have demonstrated that activation of human PBMNC leads to increased expression of FOXP3 in most $CD4^+$ and $CD8^+$ T-cells. [32] In PBMNC from both patient IV.1 and the normal control, activation led to increased FOXP3 mRNA expression (data not shown), but levels in the patient's cells remained persistently low relative to the normal control (approximately 40%) (Figure 3A). The observed increase in FOXP3 message with activation of the patient's cells suggested that at least some of the sequences required for inducible mRNA expression were present. It did not, however, rule out the possibility that some regulatory sequences required for optimal expression were altered or that mRNA stability was affected. To investigate the possibility of a defect in an upstream, non-coding region of the *FOXP3*-gene, we amplified and sequenced approximately 10-kb upstream of the first coding exon (exon 1) to the transcriptional start site of the next encoded gene, GAGE1 (Figure 3B). This revealed a 1388-bp deletion (g.del–6247_–4859) (numbering per den Dunnen and Antonarakis (36)) encompassing the 3′ half of the untranslated exon -1 and extending downstream more than 1 kb into the adjacent intron (Figure 3C). This novel mutation was also present in patient IV.2 but not in more than 100 normal X-chromosomes or in clinically normal males (patients II.3 and II.10) from this kindred (data not shown). The large deletion made it possible to develop a rapid PCR-based screening test to evaluate quickly other members of this kindred and to identify carrier females. Specific primers were designed to regions flanking the deletion to amplify a product of 1805 bp from the normal allele and a product of 417 bp from the mutated allele. As noted in Figure 1, the 2 affected males IV.1 and IV.2 demonstrate only the 417-bp product, whereas clinically normal males (patients II.3 and II.10) demonstrate only the larger 1805-bp product. In carrier females, both products are generated. Interestingly, all but 1 female (patient II.12) in this 4-generation kindred were

found to be carriers (Figure 1).

Effect of the Identified Mutation on FOXP3 Synthesis

The large deletion identified in this kindred encompasses the 3′ half of exon -1 and a portion of the adjacent intron leading to absence of the canonical splice donor site at the 3′ end of exon -1 (Figure 3B and C). We predicted that this deletion may prevent splicing of the FOXP3 pre-mRNA. To evaluate this possibility, PCR primers (PS 1) were designed in the 5′ portion of exon -1 and spanning the exon/intron junction at the 5′ end of exon 1 (Figure 4B).

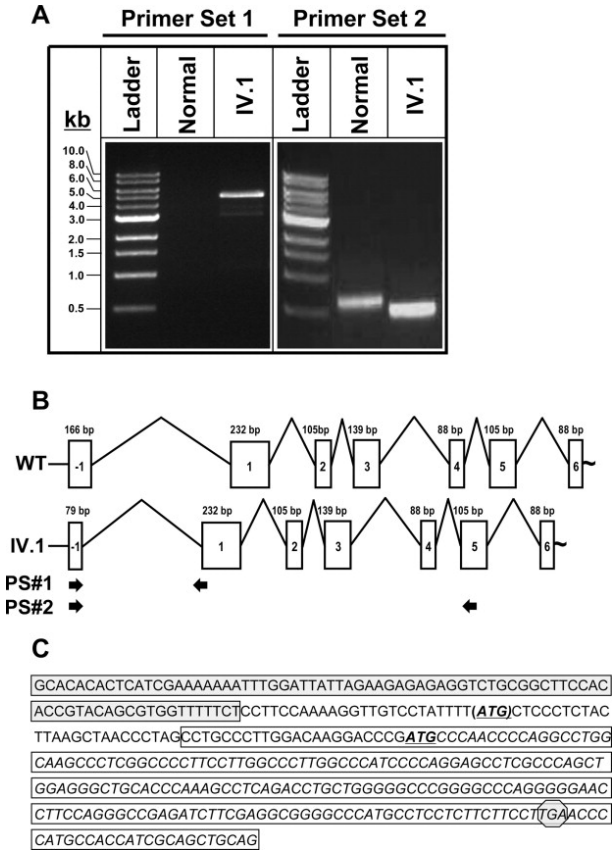

Figure 4. The identified deletion mutation leads to abnormal mRNA splicing. (A) Ethidium bromide-stained agarose gel showing FOXP3 cDNA fragments amplified from a normal control and patient IV.1. Primer set 1, capable of amplifying only unspliced pre-mRNA, demonstrates amplification of a 4880-bp fragment in patient IV.1 but not in the control (left panel). Primer set 2, which amplifies spliced mRNA, demonstrates amplification of a 793-bp fragment from the normal control and a 601-bp fragment from patient IV.1 (right panel). In each case, amplification of the fragment from patient IV.1 required the use of 10-fold more template than the normal control and an increase in PCR amplification cycles from 30 to 40. (B) Structure of the wild-type and mutant FOXP3 pre-mRNA. The position of primer set 1 (PS 1) and primer set 2 (PS 2) are shown. (C) Sequence of the 601-bp spliced mRNA fragment from patient IV.1. The remaining portion of exon -1 is shown in the shaded box, and exon 1 is shown in the unshaded box with the usual translation initiation codon shown in bold italics within exon 1. The fragment in the center is derived from the intronic region downstream of the deletion, and it is spliced to exon 1 via an alternative splice donor site. The premature translation initiation codon within this intronic fragment is shown in parentheses and bold italics. Initiation from this alternative, upstream ATG ends in a stop codon in exon 1, denoted by the shaded octagon.

These primers amplify a product only if unspliced pre-mRNA containing intron -1 is present. This primer pair will not amplify a product from spliced mRNA lacking intron -1. PCR amplification using these primers and cDNA from a normal control yielded no detectable product,

suggesting that there is little unspliced pre-mRNA under normal conditions. Amplification from the cDNA of patient IV.1 yielded a 4880-bp fragment containing the upstream portion of exon -1 and the remaining portion of intron -1 lacking the deleted fragment (Figure 4A and data not shown). This suggests that, in the patient, there is decreased efficiency of mRNA splicing leading to accumulation of unspliced pre-mRNA that may be degraded via nonsense-mediated decay. (37) Because the identified deletion included only upstream sequences of the *FOXP3*-gene, it was theoretically possible that the patient could generate wild-type protein if protein coding exons 1–11 were spliced normally. To determine whether the patient could generate any correctly spliced FOXP3 mRNA product, PCR primers (PS 2) were designed in the 5′ portion of exon -1 and in exon 5 (see Figure 4B). PCR amplification from the cDNA of a normal control readily generated the predicted 793-bp fragment corresponding to fully spliced mRNA including exons -1 through exon 5 (Figure 4A). Because of the relatively low FOXP3 mRNA expression levels in the patient's sample, amplification from the cDNA of patient IV.1 was more difficult and required the use of at least 10-fold higher cDNA template concentrations and an increase in PCR amplification cycles from 30 to 40. With these modifications, a 601-bp fragment was amplified and subsequently sequenced. This product represented spliced mRNA containing the remaining portion of exon -1 appended to a 52-bp intronic fragment derived from the sequence immediately downstream of the deletion. The 3′ end of this intronic fragment was spliced via an alternate splice donor site to an intact exon 1 (see Figure 4C). The inserted intronic fragment contained a new ATG translation initiation codon. Initiation of translation from this alternative, upstream ATG would yield an out-of-frame polypeptide of 77 amino acids that terminates with a premature stop codon near the 3′ end of exon 1 (Figure 4C). A second premature stop codon in this reading frame would be encountered in exon 3. Nonsense codons encountered upstream of spliced exon-exon junctions can target mRNA molecules for rapid degradation via nonsense-mediated decay mechanisms.(37,38) Whether this may explain the low FOXP3 mRNA levels observed in PBMNC from affected males in this kindred remains to be determined. Interestingly, the spliced product from the patient also lacked exon 2, a well-described, naturally occurring splice variant of the FOXP3 mRNA. [39]

Evaluation of Regulatory T-Cell Number and Function

To determine whether the low FOXP3 mRNA expression in PBMNC correlated with decreased FOXP3-protein expression and consequently decreased regulatory T-cell numbers, PBMNC were stained with specific antibodies to CD3, CD4, CD25, and FOXP3 and evaluated by flow cytometry. Five percent to 6% of the $CD4^+$ T-cells from normal controls demonstrated FOXP3 expression (Figure 5A), consistent with previous reports (32,40) Virtually all $FOXP3^+$ cells from the normal control were CD25high (data not shown).

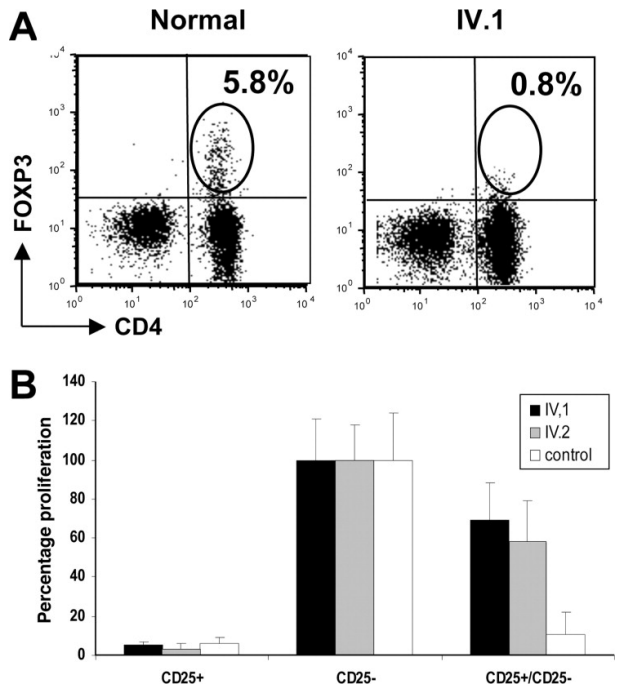

Figure 5. (A) FOXP3-protein expression in peripheral blood mononuclear cells. PBMNC from patient IV.1 and a healthy control were stained intracellular for FOXP3 and analyzed by flow cytometry for the presence of $CD4^+CD25^+FOXP3^+$ cells. Gating on $CD3^+$ cells, the normal control patient showed expression of FOXP3-protein in approximately 6% of the cells. In contrast, FOXP3 expressing cells were almost undetectable in patient IV.1. (B) $CD4^+CD25^+$ T-cells from patients IV.1 and IV.2 demonstrate decreased suppressive activity. The suppressive activity of $CD4^+CD25^+$ T-cells from patients and normal controls was tested in a co-culture system using separated $CD4^+CD25^-$ effector cells and $CD4^+$CD25high T-cells. The purified effector cells ($CD4^+CD25^-$ underwent significant proliferation upon CD3 cross-linking, whereas the purified $CD4^+$CD25high cells showed little proliferative response. In co-culture, the $CD4^+$CD25high population derived from patients IV.1 and IV.2 demonstrated markedly diminished suppressive activity compared with cells derived from a healthy normal control patient.

In contrast, less than 1% of the $CD4^+$ cells in patient IV.1 and IV.2 were $FOXP3^+$, with none being FOXP3high (Figure 5A). Interestingly, despite the low number of $FOXP3^+$ cells, the number of $CD4^+CD25^+$ cells was comparable with healthy controls in both patients (data not shown), suggesting that these cells are either regulatory T-cells that lack FOXP3 expression or are activated T-cells expressing CD25 as a marker of their activation. To evaluate whether the $CD4^+CD25^+$ cells present in the patients have regulatory function, a standard co-culture assay was performed. (30) As shown in Figure 5, $CD4^+CD25^+$ T-cells from a normal control and from 2 affected males (patients IV.1 and IV.2) did not proliferate significantly in response to cross-linking of CD3 (Figure 5B). In contrast, $CD4^+CD25^-$ effector cells proliferated strongly in response to anti-CD3 stimulation, with H3 thymidine incorporation being slightly higher in

control lymphocyte cultures compared with those of patients IV.1 and IV.2 (Figure 5B and data not shown). This is likely the result of exposure to immunosuppressive medications that each of the patients was taking at the time. Co-culture of $CD4^+CD25^+$ regulatory T-cells from the normal control with autologous responder cells demonstrated the highly suppressive effect of the $CD4^+CD25^+$ regulatory T-cells (90% suppression). In contrast, the suppressive activity of $CD4^+CD25^+$ T-cells from patients IV.1 and IV.2 was found to be markedly reduced compared with the normal control (34% and 41%, respectively), indicating altered regulatory function (Figure 5B).

Cytokine Expression Profile Within the Intestinal Mucosa

Because of the dramatic allergic phenotype in these IPEX syndrome patients, analysis of the cytokine expression profile within inflamed duodenal mucosa was performed to evaluate whether the infiltrate was more characteristic of a T-helper (Th) 1 or Th-2 response. Biopsy specimens were obtained from patients IV.1 and IV.2 before and 6 weeks after initiation of immunosuppression (steroids, tacrolimus, and azathioprine). Cytokine mRNA expression levels in the mucosal biopsy material were evaluated by semiquantitative and quantitative PCR. Prior to treatment, patients IV.1 and IV.2 had markedly increased levels of IFN-γ and IL-4 transcripts compared with the normal control patients (Figure 6), suggesting a mixed Th-1 and Th-2 cell infiltrate in the bowel mucosa.

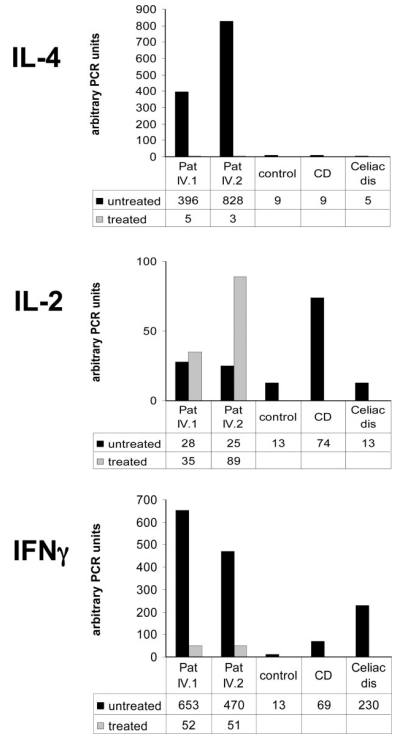

Figure 6. Real-time PCR analysis of cytokine expression profile in inflamed duodenal tissue. Th-1 (IFN-γ) and Th-2 (IL-4) as well as regulatory cytokine (IL-2) expression were analyzed before and after successful therapy in patients IV.1 and IV.2 compared with normal noninflammatory controls (n = 5) and inflammatory controls from patients with active Crohn's disease (CD, n = 5) or untreated celiac disease (n = 5). Inflamed duodenal mucosa from patients IV.1 and IV.2 demonstrates a tremendous up-regulation of IL-4 and IFN-γ, which returns to control levels following successful immunosuppressive therapy. In contrast, IL-2 expression is modest in untreated patients but increases with therapy.

With therapy, IFN-γ and IL-4 levels returned to near normal (Figure 6). In comparison, duodenal biopsy specimens from patients with other active inflammatory bowel diseases, including celiac disease and Crohn's disease, demonstrated very little IL-4 mRNA expression but increased levels of IFN-γ mRNA, suggesting primarily Th-1-mediated bowel inflammation in these diseases. Although IFN-γ is only modestly up-regulated in Crohn's disease, it is strongly up-regulated in celiac disease (Figure 6). Despite this, the mean level of IFN-γ expression in the 2 untreated IPEX syndrome patients (>550 arbitrary units) was more than 2-fold higher than in 5 active celiac disease patients, likely reflecting the intensity of inflammation in patients with untreated IPEX syndrome. Interestingly, IL-2 mRNA expression levels were significantly increased in bowel mucosa from patients with untreated Crohn's disease but were only increased in IPEX syndrome patients following immunosuppressive

therapy (Figure 6). As a control, IL-5 mRNA expression was also evaluated in the mucosal samples but was absent in both IPEX syndrome and the other inflammatory bowel conditions tested (data not shown).

3.1.5 Discussion

The family reported here presented with an atypical form of IPEX syndrome that was characterized by multiple severe food allergies associated with AIE. The patients also had eczema, hyper-IgE, and eosinophilia but, unlike most other cases of IPEX syndrome, did not present with endocrinopathy or cytopenia. The restricted symptomatology observed in the affected members of this family demonstrates the clinical heterogeneity of the IPEX syndrome and expands the range of patients for whom IPEX syndrome should be considered as a diagnosis. As suggested by this study, infants presenting with an unusually severe form of allergy early in life may require screening for IPEX syndrome. To date, no clear genotype-phenotype correlations have been reported, but atypical or milder forms of IPEX syndrome have been observed, and long-term survivors such as members of this family have been described. (17,22,41,42) Similar to other reports, (31,43) all of the female carriers identified in this kindred were healthy and had no signs of autoimmunity. Interestingly, inheritance of the mutant allele in this family appears to be skewed in the female offspring of carrier mothers (see Figure 1); 90% (9/10) of females compared with 60% (6/10) of the male offspring inherited the mutant allele. Although it is theoretically possible that gametes carrying the mutant allele are more successful at fertilization or more viable than their wild-type counterparts, there is no evidence that FOXP3 is expressed at any time during fertilization or development. We believe that this difference is most likely a chance occurrence. This is supported by the inheritance observed in a very large kindred with IPEX syndrome initially described by Powell et al (17) and Bennett et al. (31) Currently in this kindred, 65.2% (15/23) of females born to carrier mothers have inherited the mutant allele, and 60.5% (23/38) of males have the mutation (data not shown). These numbers are consistent with the 60% rate of inheritance seen in the males of the kindred presented here and are close to the expected frequency of 50%. Sequence analysis revealed a novel mutation in an upstream non-coding region of the *FOXP3*-gene (Figure 3B). The mutation consists of a large deletion involving the 3′ half of the untranslated exon -1 and more than 1 kb of intron -1 of the *FOXP3*-gene, resulting in the loss of 1388-bp of genomic DNA. Because the deletion affects only the untranslated portion of the gene, it is theoretically possible that the patients could express normal FOXP3 mRNA and protein. Unfortunately, the deletion leads to loss of the usual splice donor site at the 3′ end of exon -1 and, consequently, decreased efficiency of pre-mRNA splicing as evidenced by the accumulation of unspliced pre-mRNA (Figure 4A). The only spliced mRNA product amplified from the patients included an insertion of a 52-bp intronic fragment from intron -1. This intronic fragment introduces a new translation initiation codon upstream of the endogenous ATG

that would generate a 77-amino acid out-of-frame polypeptide terminating in a premature stop codon within exon 1. Both the unspliced and alternatively spliced mRNA products are predicted to meet criteria for rapid degradation via nonsense mediated decay mechanisms,37 although this has not been directly tested. Functional analyses using a co-culture system of $CD4^+CD25$high T-cells with $CD4^+CD25^-$ effector cells revealed a marked lack of suppressor function by CD25high T-cells from both patients compared with normal controls. It is important to note that both patients IV.1 and IV.2 were under tacrolimus-based immunosuppression at the time of analyses. Tacrolimus might impact on the function of regulatory T-cells. However, control experiments with lymphocytes from tacrolimus-treated patients indicate that the effect on T-cell proliferation is extremely weak under the experimental conditions used in this study (data not shown). A second important point in the interpretation of the functional data is the fact that other FOXP3-negative T-cells with regulatory functions exist, such as Th-3 or Tr1 cells, characterized by the production of transforming growth factor β and IL-10, respectively. (44) To date, it is not really clear of what origin these cells are and what their respective regulatory roles are. $CD4^+CD25$high cells were present in both patients at levels similar to healthy donors, and FOXP3-protein was not detectable in this subset of T-cells. These findings suggest that this mutation, involving a region upstream of the coding exons, results in the absence of functional FOXP3-expressing regulatory T-cells. As a consequence, uncontrolled T-cell responses occurred, exemplified by the high levels of IFN-γ and IL-4 mRNA expression by cells infiltrating the inflamed bowel mucosa in the untreated patients (Figure 6). The simultaneous activation of both Th-1 and Th-2 responses in patients IV.1 and IV.2 prior to immune suppression seems plausible in light of the clinical picture of autoimmune GI disease (Th-1 mediated) and severe food allergies (Th-2 mediated). Under normal conditions, however, Th-1 and Th-2 T-cell responses are thought to be mutually exclusive, suggesting that in the case of IPEX syndrome, absence of $FOXP3^+$ Tregs permits global activation of all effector T-cell populations. The importance of $FOXP3^+$ Tregs in the control of Th-1 effector cell activity and the maintenance of tolerance to self antigen has been established (45) and is exemplified in our patients by the observation that IFN-γ levels in the inflamed intestinal mucosa prior to treatment were more than 2-fold higher than those observed in active celiac disease, which is itself characterized by a massive Th-1, IFN-γ-mediated immune response (Figure 6). A role for regulatory T-cells in the control of Th-2 responses and tolerance to oral antigens has been postulated for some time. (46,47) Recent murine studies have demonstrated that $CD4^+CD25^+$ Tregs are required for the induction of oral tolerance (48,49) and that administration of oral antigen dramatically induces antigen-specific $CD4^+CD25^+$ Tregs numbers and function. (50,51,52) Some evidence suggests that, in addition to the usual thymus-derived Tregs population, the antigen-specific Tregs induced by oral antigen may be derived in the periphery from $CD4^+CD25^-$ T-cells. (53) Karlsson et al (11) have recently shown in humans that resolution of cow's milk allergy is associated with increased num-

bers of circulating $CD4^+CD25^+$ T-cells and decreased in vitro proliferative responses of bulk PBMNCs to bovine β-lactoglobulin. The in vitro proliferative responses to β-lactoglobulin increased more than 4-fold when $CD25^+$ cells were depleted from the PBMNCs, suggesting a direct link between the increase in $CD4^+CD25^+$ Tregs and resolution of the cow's milk allergy. (11) The inflammatory mucosal lesions observed in both patients showed a combination of severe villous atrophy and massive mucosal mononuclear cell infiltration (Figure 2). This infiltrate consists predominately of T-lymphocytes and is a main feature of AIE. Most often, villous atrophy is associated with crypt hyperplasia. (54) Total villous atrophy on duodenal biopsies can initially mislead to suspect celiac disease. However, in celiac disease, villous atrophy is associated with a striking increase in the number of intraepithelial lymphocytes. (55) In contrast, T-cell infiltration in AIE is predominantly observed in the lamina propria, with no or only moderate increase in intraepithelial lymphocytes. (56) Whereas in celiac disease a marked increase in T-lymphocytes expressing the T-cell receptor of the $\gamma/\delta+$ type was observed, (57) in AIE, T-cell receptor expression is restricted to the $\alpha/\beta+$ subset. In some patients with AIE, villous atrophy is associated with massive epithelial cell apoptosis and crypt abscess formation, as seen in our patients (Figure 2). Auto-antibodies of the anti-enterocyte or anticolonocyte type are a hallmark of AIE, and these antibodies are present in the vast majority of IPEX syndrome patients. (58,59) Antienterocyte antibodies are most often of IgG origin, but auto-antibodies of the IgA or IgM isotype were also described in a subgroup of patients. (60) Indirect immunofluorescence studies showed that these auto-antibodies are directed against components of the cytoplasm of mature enterocytes, with an increasing intensity toward the villus tip. Positive staining of the intestinal brush border membrane can also be observed. A pathogenic role in the onset of intestinal inflammation—as suspected in the past—is unlikely. They are rather an epiphenomon of intestinal inflammation. A precise kinetic study performed in one patient showed that auto-antibodies occurred after the onset of intestinal lesions. (61) Another, highly specific antibody often detected in IPEX syndrome patients is directed against a gut- and kidney-specific 75-kilodalton antigen named AIE75. (27,62) This antigen shares over 99% identity with NY-Co-38, a colon cancer-related auto-antigen. (63). Interestingly, in 6 of the 8 affected boys in this family, the onset of severe diarrhoea occurred within the first 8 weeks of life. A major change in the type of alimentation, such as switching from mother's milk to a cow's milk-based infant formula preceded clinical symptoms in some of the patients. In patients IV.1 and IV.2, this was associated with the appearance of specific IgE at very high titers against cow's milk protein (F2 RAST highly positive, data not shown). A marked worsening of symptoms has been reported in other patients with IPEX syndrome following immune activation by either immunization or infections.(17) Additionally, Zahorsky–Reeves and Wilkinson (64) have recently shown that scurfy mice whose T-cells bear a monospecific T-cell receptor (Rag1-/- sf/Y Ova) require antigen (ovalbumin) exposure to trigger scurfy disease and unmask the regulatory T-cell de-

fect. Similar to ovalbumin in this mouse model, antigenic epitopes from cow milk proteins appear to initiate immune activation in these patients and precipitate progression of disease. It is not known whether the same antigen is capable of simultaneously inducing both Th-1 and Th-2 responses or if different antigens are required. The prominence of GI and skin disease in IPEX syndrome and particularly in the patients reported here suggests an important role for Tregs at interfaces that are unrelentingly exposed to exogenous antigens such as bacterial products and alimentary antigens. Recent data suggest that $CD4^+CD25^+$ Tregs express receptors of the innate immune system including Toll-like receptors 2, 4, 5, 7, and 8, enabling them to sense bacterial products. (65,66) In vitro stimulation of purified murine $CD4^+CD25^+$ T-cells by activation of Toll-like receptor 4 with bacterial lipopolysaccharide increased the suppressor efficiency of these cells, (65) whereas activation of Toll-like receptor 2 decreased the suppressive activity. (66) Ongoing studies to clarify further the precise role of Tregs at these important self/environment interfaces will help to elucidate the pathogenesis of IPEX syndrome and may provide opportunities for more targeted therapeutic interventions. In summary, IPEX syndrome is a more heterogeneous disorder than previously recognized, exemplified by this family demonstrating a form of the syndrome combining autoimmunity and severe allergy. We were able to demonstrate in our patients that a large deletion in an upstream noncoding region of *FOXP3*-gene causes a defect in mRNA splicing. Subsequently, FOXP3 mRNA and protein levels are almost undetectable, causing profound Tregs dysfunction. This presents clinically as a combined Th-1/Th-2 disorder, as exemplified by the cytokine mRNA profile within the inflammatory intestinal mucosa. This observation gives new insight into the physiopathology of immunoallergic dysregulation, opening new diagnostic and potentially therapeutic avenues.

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3.2 Reduced Expression of FOXP3 and Regulatory T-cell Function in Severe Forms of Early-Onset Autoimmune Enteropathy

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Background & Aims
Little is known about the pathophysiology of early onset forms of autoimmune enteropathy (AIE). AIE has been associated with mutations in FOXP3—a transcription factor that controls regulatory T-cell development and function. We analyzed the molecular basis of neonatal or early postnatal AIE using clinical, genetic, and functional immunological studies.

Methods
Gastroenterological and immunological features were analyzed in 9 boys and 2 girls with AIE that began within the first 5 months of life. FOXP3 and IL2RA were genotyped in peripheral blood monocytes. FOXP3 messenger RNA and protein expression were analyzed using reverse-transcription polymerase chain reaction, flow cytometry, and confocal immunofluorescence of $CD4^+$ T-cells. Regulatory T-cell function ($CD4^+CD25^+$ was assayed in co-culture systems.

Results
AIE associated with extra-intestinal autoimmunity was severe and life-threatening; all patients required total parenteral nutrition. Regulatory T-cells from 7 patients had altered function and *FOXP3*-mutations that resulted in lost or reduced FOXP3-protein expression; 2 infants had reduced regulatory T-cell activity and reduced levels of FOXP3-protein, although we did not detect mutations in FOXP3 coding region, poly-A site, or promoter region (called *FOXP3*-dependent AIE). Two patients had a normal number of regulatory T-cells that expressed normal levels of FOXP3-protein and normal regulatory activity in in vitro co-culture assays (called *FOXP3*-independent AIE). No mutations in IL2RA were found.

Conclusions
Most cases of AIE are associated with alterations in regulatory T-cell function; some, but not all, cases have mutations that affect FOXP3 expression levels. Further studies are needed to identify mechanisms of AIE pathogenesis.

3.2.1 Introduction

The term autoimmune enteropathy (AIE) comprises a variety of different intestinal disorders with variable clinical expression, ranging from mild and isolated gastrointestinal to severe systemic diseases. [1,2] In contrast to late-onset adult forms, early forms of AIE most often start within the first months of life and have a particularly severe evolution. To date, few molecular approaches exist to elucidate the pathogenesis of AIE. Neonatal or early postnatal onset of a chronic disease might indicate a genetic disorder. This hypothesis was recently confirmed by the discovery of disease-causing mutations in the *FOXP3*-gene [3] , [4,5] in boys with a particularly severe systemic form of AIE, a disorder now called immune dysregulation, polyendocrinopathy, autoimmune-enteropathy X-linked syndrome (IPEX; OMIM304930). In patients with IPEX, AIE is often combined with insulin-dependent diabetes mellitus and hematological autoimmune manifestations. [6] We recently described a different clinical picture of the IPEX syndrome associating AIE and severe immune-mediated allergic skin and gastrointestinal manifestations. [7] Since the discovery of disease-causing mutations in FOXP3 (located on Xq11.23-13.3), a transcription factor essential to development and function of regulatory T-cells, [8,9] the analysis of functional abnormalities of regulatory T-cells became central to the search for causes of autoimmune diseases. Most data available for FOXP3-related AIE were collected from boys. However, there are also descriptions of severe forms of AIE in girls, indicating *FOXP3*-independent forms. So far, no studies of the function of regulatory T-cells were performed in these patients. In the present study, we aimed to analyze the clinical presentation and molecular basis of severe forms of early-onset AIE and their evolution. We restricted our analysis to a group of patients (boys and girls) starting AIE within the first 5 months of life. All patients had a life-threatening course and required total parenteral nutrition combined with heavy immunosuppression for initial stabilization of their course. Molecular analyses included *FOXP3*- and *IL2RA* genotyping, FOPX3- and CD25 protein expression, as well as analysis of regulatory and effector T-cells in all 11 patients.

3.2.2 Material and Methods

Patients/Healthy Controls

Eleven patients (9 boys, 2 girls) with AIE were included in this study. All patients were on immunosuppressive therapy when fresh peripheral blood mononuclear cells (PBMNC) were collected, no samples of untreated patients prior to diagnosis were available. All blood tests were performed before bone marrow transplantation. Upon informed parental consent, a maximal amount of 10 mL blood was harvested from all patients and from healthy children ($n = 6$). At the time of blood draw, all patients were in a clinically stable situation (with complete or close to complete remission) and under heavy immunosuppression combining steroids (0.3-1.5 mg/kg), azathioprine (2-3 mg/kg), and tacrolimus (residual levels of 6-12

ng/mL) or rapamycin (residual level of 5-10 ng/mL). Co-culture experiments were performed under stable rapamycin-based medication for patients 1, 2, 8, and 11, and stable tacrolimus-based medication for patients 3, 4, 5, 6, 7, 9, and 10. In patients 1, 2, 5, 8, and 11, cells were tested while patients were under tacrolimus and subsequently rapamycin-based immunosuppressive therapy, with comparable results (data not shown). However, under rapamycin medication, absolute proliferation rates were markedly lower, making these analyses more difficult. Four non-AIE patients receiving rapamycin- (n = 2) or tacrolimus- (n = 2) based immunosuppression for small bowel transplantation were included as treatment controls. Experimental conditions were initially optimized with blood samples from healthy adult donors, obtained from the "Centre National de Transfusion Sanguine," Paris, France. This study was fully approved by our local ethics committee (CPP II Ile de France 2009-155, PHRC AOM08087).

Cell Isolation and Culture

PBMNC were isolated on Ficoll (mononuclear cell separation medium; Eurobio, France) density (1077 $\pm$ 0.001) and cultured in RPMI Glutamax 1640 medium (Invitrogen, Cergy Pontoise, France) according to standard protocols. Cells were stimulated with soluble anti-CD3 (UCHT1 1 μg/mL) and anti-CD28 antibodies (0.5 μg/mL) (BD Pharmingen, Le Pont de Claix, France) with or without recombinant interleukin (IL)-2 (40 IU/mL) for up to 72 hours (R&D Systems, Lille, France). Supernatants were collected and cytokine production was analyzed by enzyme-linked immunosorbent assay (interferon [IFN]-γ and IL-17, Duoset kits, IL-13 high-sensitivity Quantikine kit D1300; R&D Systems). To obtain polyclonal $CD4^+$ T-cell lines, $CD4^+$ cells were negatively selected from PBMNC by magnetic cell sorting (MACS; Miltenyi Biotec, Paris, France), as described here, and stimulated with phytohemagglutinin-L (1 μg/mL; Sigma, St Quentin Fallavier, France) and IL-2 (40 IU/mL) and maintained in IL-2 (40 IU/mL) for a period of 4 to 6 weeks before restimulation by anti-CD3 and anti-CD28 $\pm$ IL-2 for 72 hours. For flow cytometric studies, conjugated antibodies directed against CD3, CD4, CD25, CD8, CD56, CD127, CD14, and CD19 (all from BD Pharmingen) were used. FOXP3 staining of $CD4^+CD25^+$ cells and, since 2007, of $CD4^+CD25^+CD127dim$ cells, was performed using rat monoclonal anti-human FOXP3 antibody (clone PCH101, eBioscience distributed by Clinisciences, Montrouge, France), according to manufacturer's instructions.

Genotypic Analysis of the Patients

Genomic DNA was isolated from whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Courtaboeuf, France) according to manufacturer's protocol. Each of the 11 coding exons of FOXP3, including intron/exon boundaries and the untranslated 3′ UTR region surrounding the first polyadenylation signal were amplified from genomic DNA by polymerase chain reaction (PCR) using intronic primer pairs as reported previously. [5,11] PCR products were

separated by 1% agarose gel electrophoresis, purified with the QIAquick PCR Purification Kit (Qiagen) and directly sequenced using the BigDye Terminator Cycle Sequencing Kit PE (Applied Biosystems, Courtaboeuf, France). In patients without *FOXP3*-mutations, the entire IL2RA gene was sequenced in a similar manner.

Real-time PCR Analysis of FOXP3 Messenger RNA

Total RNA extracted from 5×106 fresh PBMNC was used to analyze FOXP3 messenger RNA (mRNA) expression as described [7] using the following primers for FOXP3 5′-CCCACAAGCCAGGCTGAT-3′ and 5′-GCATCGGGTCCTTGTCCA-3 and a commercially available Taqman assay for the housekeeping gene RPLP0 (Applied Biosystems, Carlsbad, CA).

Confocal Microscopy

The cellular localization of FOXP3 (nuclear or cytoplasmic) was analyzed using an LSM 510 ZEISS laser scanning confocal microscope (Carl Zeiss Inc., Le Pecq, France). A total of 250,000 cells from $CD4^+$ polyclonal T-cell lines (controls or IPEX patients) were collected by centrifugation on glass slides and fixed with acetone. Cells were labeled with Affinity-purified rat anti-human FOXP3 (clone PCH101) or affinity-purified rat IgG2a isotype as a control (BD Pharmingen), and staining was revealed using fluorescein isothiocyanate-coupled secondary goat anti-rat IgG (H+L) antibody (Abcam, Paris, France). Nuclei were stained with Topro-3 (Molecular Probes, Interchim, Montluçon, France) after treatment with RNAse A (Sigma). Preparations were mounted using Vectashield mounting medium (Vector Laboratories, distributed by ABCYS, Paris, France). To precisely localize a positive fluorescence signal, cells were analyzed in a 3-dimensional manner and a final merge was performed to confirm nuclear localization of FOXP3.

Co-culture Experiments of $CD4^+CD25^+$ and $CD4^+$ $CD25^-$ T Cells

$CD4^+CD25^+$ T-cells were isolated from PBMNC by magnetic cell sorting combining 2 steps. $CD4^-$ cells were first selected using a cocktail of biotin-conjugated monoclonal anti-human antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, T-cell receptor γ/δ and glycophorin A, and microbeads conjugated with monoclonal anti-biotin antibody (clone Bio3-18E7.2; $CD4^+CD25^+$ regulatory T-cell isolation kit; Miltenyi Biotec, Paris, France). This negative selection step resulted in a >95% pure population of $CD4^+$ cells, which was then positively sorted for $CD25^+$ cells using microbeads conjugated with monoclonal anti-CD25 antibody (clone 2A3). Magnetic separation was performed on 1 LD column for the negative and 2 MS columns for the positive selection process, respectively, according to manufacturer' s instructions (Miltenyi Biotec). The nonretained $CD4^+CD25^-$ and the selected $CD4^+CD25^+$

cells were, respectively, 85% and 85%-90% pure, as assessed by flow cytometry. To evaluate the suppressor activity of $CD4^+CD25^+$ T-cells, $CD4^+CD25^-$ cells (50 000 cells/well) were cocultured with $CD4^+CD25^+$ regulatory T-cells or control $CD4^+CD25^-$ cells at a ratio of 1:0, 1:2, or 1:5 suppressor to responder cells in the presence of coated anti-CD3 antibody (UCHT1 1 μg/mL; Becton Dickinson). After 5 days, cells were pulsed with 3H-thymidine for 18 hours and incorporation was measured on a Wallac 1450 microbeta/Trilux counter (Perkin Elmer, Courtaboeuf, France). Suppressor activity of $CD4^+CD25^+$ cells was determined as the percent of inhibition between the proliferation rate of anti-CD3-stimulated $CD4^+CD25^-$ cell co-cultured with $CD4^+CD25^+$ cells and of $CD4^+CD25^-$ cells cultured alone.

Statistics

Data are presented as means of at least 3 independent experiments. The nonparametric Mann–Whitney test was used to compare different groups. P values < 0.05 were considered statistically significant.

3.2.3 Results

Clinical Features

Clinical presentation of all patients is summarized in Table 1.

Table 1. Clinical Characteristics and Evolution of Patients with Autoimmune Enteropathy

Patient no./sex	1/M	2/M	3/M	4/M	5/M	6/M	7/M	8/M	9/M	10/F	11/F
Age of onset	5 wk	3 wk	Birth	Birth	6 wk	4 wk	Day 7	4 wk	4 mo	4 mo	3 mo
Main symptoms	Enteropathy	Enteropathy	Enteropathy	Enteropathy	Enteropathy	IDDM	Enteropathy	IDDM	Enteropathy	Enteropathy	Enteropathy
	Eczema	Eczema	TCP	Eczema	Eczema	Enteropathy	IDDM	Enteropathy			TCP
Colonic involvement	+	+	+	+	+	No	+	No	+	+	+
Endocrinopathies											
IDDM	No	No	No	No	No	Yes	Yes	Yes	No	No	No
Other	No	No	Basedow hyper-thyreoiditis	No	Hypo-thyreoiditis	No	No	No	Hypo-thyreoiditis	No	No
Other organs	Skin	Skin, HP gastritis	Skin, HP gastritis	No	Interstitial nephritis	Skin, AI-hepatitis	Skin	Skin	AI-deathness	Skin	AI-pericarditis
Hematologic symptoms	No	No	TCP	Hemolytic anemia TCP	Hemolytic anemia	Hemolytic anemia Agranulo-cytosis	Hemolytic anemia	No	No	Neutropenia	TCP
Allergy	Yes, eosinophilia	Yes, eosinophilia	Yes, eosinophilia	Yes, eosinophilia	Yes, eosinophilia	No	No	Yes	No	Yes	Yes
IgE	3000	11,000	5500	8500	12,500	2150	650	1500	–	350	400
AEA	+	+	+	+	+	+	+	+	+	+	–
AIE75	+	+	+	+	+	+	+	+	–	–	–
Immunosuppression	Tacro> rapamycin	Tacro> rapamycin	Tacrolimus	Tacro> rapamycin	Tacro> rapamycin	Tacrolimus	Tacrolimus	Tacro> rapamycin	Tacrolimus	Tacrolimus	Tacro> rapamycin
BMT	No	No	Yes	Scheduled	Yes at 10 y	Planned	Planned	No	No	No	No
Outcome	Stable	Stable	Death, severe GvH	Death during induction therapy Multiorgan failure	Stable after BMT	Death, cardiac arrhythmia	Death, sepsis	Stable	Stable	Stable	Stable
Age (01/2010)	9 y	6 y	Death at 8 y	Death at 14 mo	10 y	Death at 8 m	Death at 7 mo	9 y	18 y	7 y	5 y

AEA, anti-enterocyte autoantibodies; AIE75, anti-autoimmune enteropathy 75 kDa antibodies; AI, autoimmune; BMT, bone marrow transplantation; F, female; GvH, graft-vs-host reaction; HP, *Helicobacter pylori*; IDDM, insulin-dependent diabetes mellitus; M, male; Tacro, tacrolimus; TCP, thrombocytopenia.

No patient was issue of a consanguineous family, and in 2 families other members were affected with IPEX syndrome. Mean age at onset of AIE was 10.3 $\pm$ 5 weeks (median 4 weeks). All children had an extremely severe form of AIE and required total parenteral nutrition with

repeated albumin and erythrocyte substitutions. Small bowel involvement with superficial and/or deep ulcerations was observed in all patients at diagnosis, and 9 patients also displayed colonic inflammation. All 11 children displayed various additional extra-intestinal autoimmune symptoms (Table 1). All patients were initially stabilized by the use of heavy immunosuppression combining steroid-pulses and tacrolimus, and were subsequently switched to rapamycins. [12] Symptoms of 6 patients could be controlled by rapamycin/azathioprine medication, while 5 patients did not remain in remission and were scheduled for bone marrow transplantation.[13]

FOXP3 Genotyping and mRNA/Protein Expression

A complete FOXP3 genotyping, including the poly-A region was performed in all 11 patients. Mutations in the *FOXP3*-gene were identified in 7 of 9 boys and none of the 2 girls (Table 2): 3 patients carried missense mutations in one of the coding exons of FOXP3, whereas 4 boys had deletions.

Table 2. Genotyping of *FOXP3* and Consequences on FOXP3 Protein Expression

Patient no.	Mutation *FOXP3*	Exon	Mutation type	Protein
1	Yes	1	g.4859-6247 del	No protein expression
2	Yes	1	g.4859-6247 del	No protein expression
3	Yes	4	g.560 c>t	P187L
4	Yes	10	g.1121 t>g	F374C
5	Yes	7	751-753 del gag	E251 del
6	Yes	7	751-753 del gag	E251 del
7	Yes	10	c. 1015 c>g	P339A
8	None		WT/Y	
9	None		WT/Y	
10	None		WT/WT	
11	None		WT/WT	

A large deletion in a non-coding region upstream of exon 1 was observed in 2 brothers (patients 1 and 2), resulting in altered RNA splicing and RNA instability. [7] Of note, 2 unrelated patients (5 and 6) showed an identical mutation in exon 7 leading to an in-frame deletion of 1 residue (E251 del). None of the mutations was observed in a cohort of $>$200 healthy controls, allowing exclusion of polymorphisms. In addition, all mutations in FOXP3 observed had marked consequence on FOXP3-protein domains as described in Table 2. In the next step, FOXP3 mRNA and protein expression were analyzed in all patients. FOXP3 mRNA was undetectable by real-time PCR in patients 1 and 2, both with a large deletion upstream of exon 1, but was detectable in all other patients at reduced or subnormal levels comparable to healthy controls or control patients treated with rapamycin (data not shown). Flow cytometric analyses of fresh PBMNC (Figure 1) revealed that 4 of 7 boys with FOXP3 deficiency had no or $<$ 1% $CD4^+CD25^+FOXP3^+$ cells (2 patients with absent mRNA and 2

patients with markedly reduced mRNA levels). Furthermore, 2 other IPEX patients, as well as 2 patients with FOXP3wt displayed reduced proportion of $FOXP3^+$ cells (2.3% and 4.2%; normal range, 5%-8%) and reduced levels of protein expression (mean fluorescence intensity 37 and 30), whereas 5%-8% $FOXP3^+$ cells were observed in the remaining 3 patients, similar to healthy controls. The intensity of FOXP3-protein expression was markedly (3- to 5-fold) reduced in $CD4^+CD25high$ cells in all patients with mutations in FOXP3, but also in 2 patients with FOXP3wt compared to controls (Table 3). Since 2007, we routinely perform FOXP3 staining in $CD4^+CD25^+CD127dim$ cells, which allowed distinguishing of activated CD4 cells, which express high CD127 levels from regulatory cells that are CD127dim. Unfortunately, this could not be verified in the deceased patients.

Table 3. Regulatory T-Cell and FOXP3 Expression in Autoimmune Enteropathy Patients

	% $CD4^+CD25^+$ cells (%)	% $CD4^+FOXP3^+$ cells (%)	FOXP3 Mean fluorescence intensity
Controls	5–10	6–9	98–158
1	6	<1	1
2	9	<1	1
3	15	8	61
4	7	3	34
5	9	<1	55
6	7	<1	30
7	7	3.3	31
8	10	4.2	30
9	9	7.4	107
10	8	5.9	89
11	11	2.3	37

To analyze FOXP3-protein location, we took advantage of the fact that this protein is up-regulated by T-cell activation. [14,15] We examined its expression in polyclonal $CD4^+$ T-cell lines obtained from patients and controls. Consistent with data from fresh PBMNC, no FOXP3-protein was observed in T-cell lines from patients 1 and 2 (g.4859-6247 del). FOXP3-protein staining was almost exclusively nuclear in $FOXP3^+$ cells from healthy controls and from all AIE except patient 7, in whom a predominantly cytoplasmic and perinuclear staining was observed (Figure 2). This patient had a mutation at the N-terminal part of exon 10 (P339A) coding for a region known to be critical for the nuclear import of FOXP3.

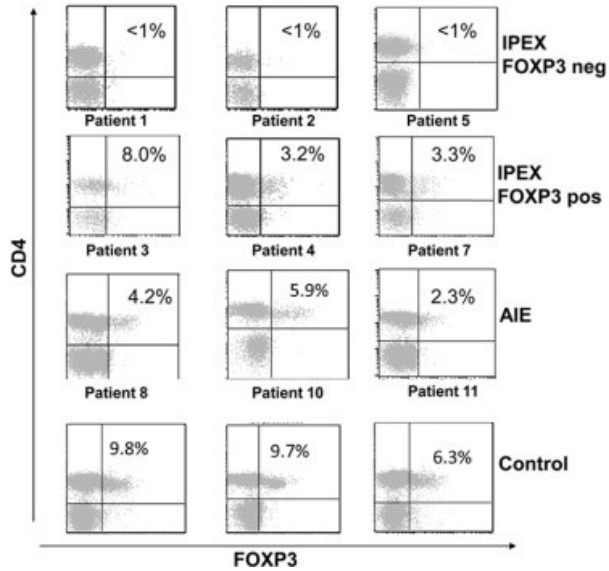

Figure 1. FOXP3 expression. Flow cytometric analysis of FOXP3-protein expression in peripheral blood mononuclear cells of autoimmune enteropathy (AIE) patients in comparison to healthy controls. Within the T $CD3^{+}CD4^{+}$ gate, 6%-10% of cells from healthy individuals stained positive for FOXP3. Immune dysregulation, polyendocrinopathy, autoimmune-enteropathy X-linked syndrome (IPEX) patients (mutated FOXP3) had either no or close to zero protein expression (patients 1, 2, and 5), or a subnormal protein expression (patients 3, 4, and 7). In *FOXP3*-independent forms of AIE, FOXP3 (patients 8, 10, and 11) expression was found to be reduced to normal. Fluorescence-activated cell-sorter analyses of patients 6 (low) and 9 (high expression) are not shown.

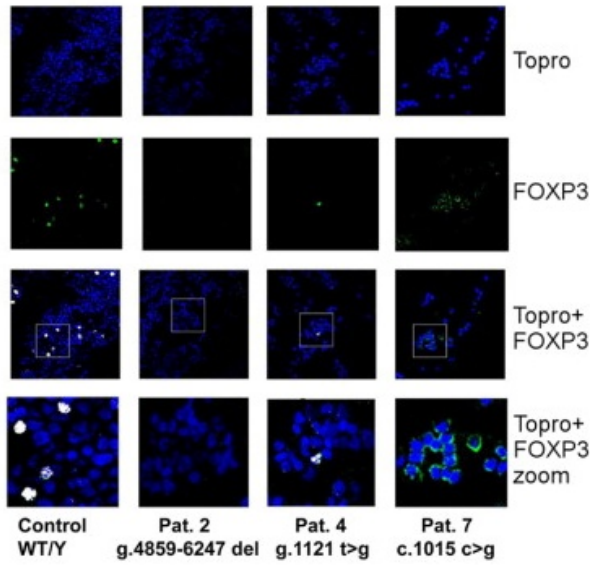

Figure 2. FOXP3-protein expression and location was analyzed by confocal microscopy in polyclonal $CD4^+$ T-cell lines from children with IPEX syndrome and healthy controls. Fluorescence images show nuclear staining with Topro in blue. FOXP3-protein appears in green with a clear white signal upon merge of the 2 fluorescent dyes indicating nuclear location. Healthy controls display a typical nuclear staining of FOXP3 (strong white signal after merge), whereas in patient 2 FOXP3 staining was completely negative and patient 4 had very few T-cells with a weak nuclear expression of FOXP3-protein. In contrast, a strong positive FOXP3 staining was observed in patient 7; however, only perinuclear and cytoplasmic FOXP3-protein locations were identified in this patient. FOXP3-protein expression correlated with the presence of messenger RNA in these experiments (data not shown).

IL2RA Genotyping and Protein Expression

Because no mutation in the *FOXP3*-gene was observed in 4 patients with early-onset AIE (2 boys and 2 girls), we tested the possibility of mutations in the *IL2RA*-gene, as recently reported in 2 patients. [16,17] Sequencing of the entire gene failed to reveal a mutation in these patients and flow cytometric analyses demonstrated normal or even up-regulated IL2RA expression (data not shown). In addition, T-cells from these patients responded to exogenous IL-2 stimulation, excluding a major defect of IL-2-IL2RA interaction.

Regulatory and Effector T-cell Functions

FOXP3 is crucial for normal regulatory T-cell development and function. In all patients with AIE, the regulatory function of $CD4^+CD25^+$ T-cells was assessed by measuring their capacity to inhibit the in vitro proliferation of $CD4^+CD25^-$ effector T-cells stimulated with anti-CD3

antibody. Under these experimental conditions, inhibition by $CD4^+CD25^+$ regulatory T-cells from healthy individuals was strong, ranging from 75%-90% (Figure 3).

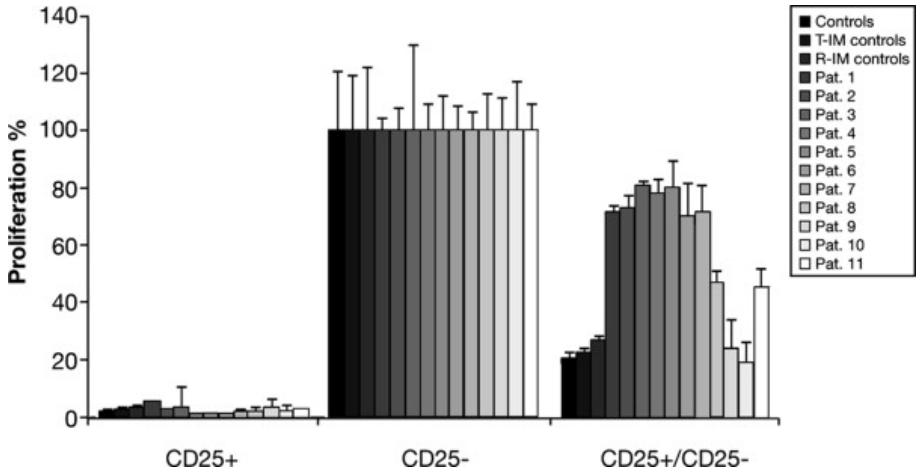

Figure 3. Function of regulatory T-cells. The suppressive activity of $CD4^+CD25^+$ T-cells from patients and healthy controls was tested in a co-culture system using purified $CD4^+CD25^-$ effector cells and $CD4^+CD25^+$ T-cells at a ratio of 5:1. Effector T-cells showed significant proliferation upon CD3 cross-linking in contrast to purified $CD4^+CD25^+$ cells. Under co-culture conditions, $CD4^+CD25^+$ cells derived from patients with a mutation in FOXP3 (patients 1-7) demonstrated markedly diminished suppressive activity compared to normal healthy controls. Patients 9 and 10 displayed normal regulatory functions, whereas patients 8 and 11 showed defective suppression, but less compared to patients with a mutation in FOXP3. Control samples from non-IPEX patients under rapamycin-based medication (R-IM, n = 2) or tacrolimus-based medication (T-IM, n = 2) revealed a normal inhibitory function of regulatory cells. Mean of 2 or 3 independent experiments for each patient is shown.

In contrast, $CD4^+CD25^+$ cells from patients with *FOXP3*-mutations displayed defective regulatory function (maximum inhibition rate of 20%-30%). $CD4^+CD25^+$ T-cells from 2 patients without *FOXP3*-mutations, but reduced FOXP3 expression (patients 8 and 11) also had a reduced inhibitory activity (inhibition rate of 55%-60%), whereas $CD4^+CD25^+$ T-cells from the 2 patients with normal FOXP3 expression (patients 9 and 10) had a normal inhibitory effect (70%-85% inhibition rate), comparable to cells from control patients on tacrolimus or rapamycin medication. *FOXP3*-mutations have been suggested to affect not only regulatory T-cell functions, but also influence effector $CD4^+$ T-cell cytokine production.18 To test cytokine production, as well as T-cell response to IL-2, fresh PBMNCs from patients and age-matched controls were stimulated with anti-CD3 and anti-CD28 antibodies $\pm$ IL-2. IFN-γ production was markedly reduced in IPEX patients, while AIE patients with FOXP3wt displayed close to normal IFN levels, despite identical immunosuppressive therapy ($P < .01$). In contrast, IL-17 secretion was significantly higher in AIE compared to IPEX patients ($P < .007$) (Figure 4).

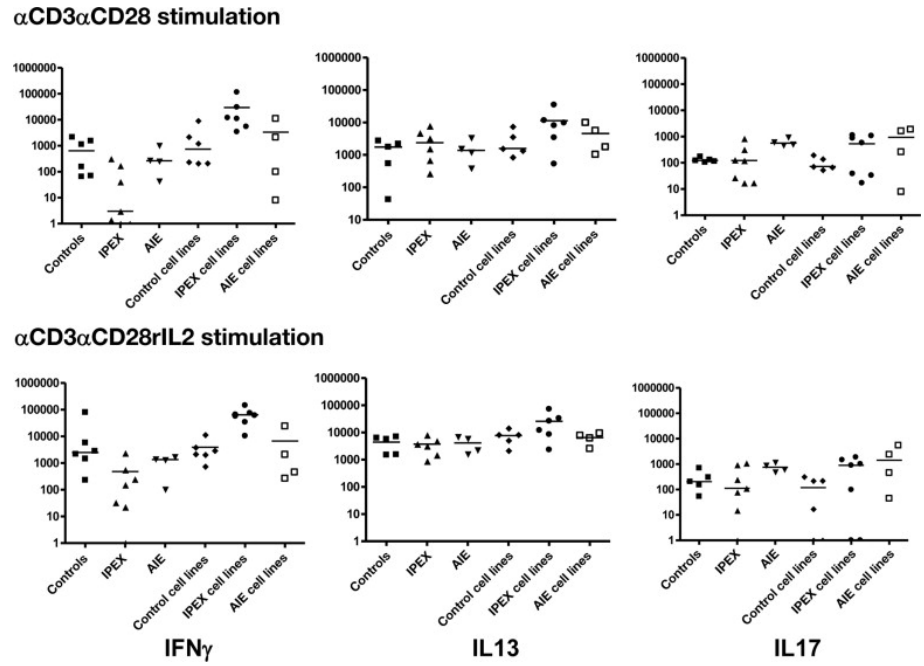

Figure 4. Cytokine production. Proinflammatory cytokine production in response to anti-CD3/anti-CD-28 ± interleukin (IL)-2 stimulation was analyzed by enzyme-linked immunosorbent assay in fresh peripheral blood mononuclear cells and $CD4^+$ T-cell lines from autoimmune enteropathy (AIE) and immune dysregulation, polyendocrinopathy, autoimmune-enteropathy X-linked syndrome (IPEX) patients and healthy controls. Results for interferon-γ (IFN-γ), IL-13, and IL-17 are shown. Each spot represents 1 individual patient and indicates the mean of 3 independent experiments.

Because all children were under heavy immunosuppressive therapy (tacrolimus or rapamycin combined to azathioprine and variable doses of prednisone), which might impair cytokine production, the same analyses were performed in parallel in polyclonal $CD4^+$ T-cell lines after 4-6 weeks of culture. As shown in Figure 3, upon subsequent re-stimulation with anti-CD3 and anti-CD28 antibodies ± IL-2, production of IFN-γ and, to a lesser degree, IL-13 and IL-17, were markedly enhanced compared to T-cell lines from controls. There was no qualitative or quantitative difference between patients with or without mutations of FOXP3.

3.2.4 Discussion

This single-center series of 11 consecutive children with early-onset AIE starting within the first 5 months of life allowed identification of a profound immune dysregulation with an altered function of regulatory T-cells in 9 of 11 patients. In 7 patients, disease-causing mutations in the *FOXP3*-gene were identified as responsible for absent or markedly reduced FOXP3-protein expression. The novel finding has been the observation that 2 infants with

early onset AIE had $CD4^+CD25^+CD127dim$ T-cells with reduced FOXP3-protein levels and regulatory activity, but no mutation was evidenced in FOXP3 coding region, its poly A site and promoter region.[19] Therefore, these patients can be considered to have a *FOXP3*-dependent IPEX-like disease. This contrasts to 2 additional AIE patients, also with wild-type FOXP3, who displayed a normal number of FOXP3 containing $CD4^+CD25^+$ T-cells with normal levels of FOXP3-protein and normal regulatory activity in in vitro co-culture assays. These patients present a *FOXP3*-independent form of AIE. The clinical phenotype associating AIE and extra-intestinal symptoms were overlapping or even identical between patients with abnormal and normal FOXP3-expression and regulatory T-cell function, respectively. However, disease progression was more severe in infants with mutated FOXP3 compared to children without mutations albeit reduced FOXP3 levels or those having a *FOXP3*-independent form of AIE. IgE levels might be a surrogate marker helping to identify less severe forms of AIE, because IgE levels were markedly higher in AIE patients with *FOXP3*-dependent compared to less severe *FOXP3*-independent forms. In this study, all patients with a mutation of FOXP3 and reduced FOXP3 levels displayed a marked dysfunction of regulatory T-cells and are therefore considered to have a *FOXP3*-dependent form of AIE. In keeping with a recent observation,[20] we did not observe any correlation between the type of *FOXP3*-mutation (genotype) and the function of regulatory T-cells. Strikingly, FOXP3-protein expression and regulatory function was impaired in $CD4^+CD25^+$ T-cells from 2 patients without mutations of FOXP3 (1 boy and 1 girl). Interestingly, the clinical course of both patients was less severe compared to patients with documented mutations in FOXP3: both patients are stable while on immunosuppressive therapy with a follow-up of several years. The reduced expression of FOXP3 mRNA and protein in AIE patients without *FOXP3*-mutations suggests that factors implicated in the regulation of FOXP3 might also contribute to the pathogenesis of these complex autoimmune diseases. Theoretically, this could happen in either a direct (upstream regulation, modified promoter function, modifier genes) or indirect manner (environmental trigger factors), as recently discussed for other transcription factors implicated in autoimmunity, such as autoimmune regulator gene. [21,22] There are interesting reports indicating that, in mice, expression of foxp3 can be regulated via different pathways, such as phosphatidylinositol 3-kinase, Akt-mammalian target of rapamycin, the latter probably linked to the sphingosine 1-phosphate receptor, a recently identified intrinsic negative regulator of regulatory T-cells. [23,24] Another possibility for the regulation of FOXP3 expression could be via proteolytic processing of FOXP325 or via epigenetic mechanisms, based on the observation of several epigenetic markers at the FOXP3 locus. In fact, methylation of CpG residues was shown to repress FOXP3 expression in murine and human cells, whereas complete demethylation is required for stable FOXP3 expression.[26] It is of importance to include these analyses in future studies, mainly of *FOXP3*-independent forms of AIE. In keeping with our description of IPEX-like but *FOXP3*-independent forms of AIE is the recent report of Caudy et al,[16]

presenting a patient with wild-type FOXP3 but CD25 deficiency due to a mutation in the *IL2RA*-gene. In our AIE patients with normal FOXP3 expression, T-cells exhibited normal or increased CD25 expression normal response to IL-2, and sequence analyses failed to detect a mutation in IL2RA. These results point to additional molecular defects that await further characterization. Both cases were sporadic, precluding a classical genetic approach. Noticeably, 2 unrelated patients with an identical mutation in exon 7 (751-753 del gag), reduced FOXP3 expression, and a clear dysfunction of regulatory T-cells, presented with different clinical forms of IPEX: 1 had severe insulin-dependent diabetes mellitus associated with AIE, whereas the other child did not develop insulin-dependent diabetes mellitus, but suffers from severe allergic reactions to drugs and dietary proteins. These observations emphasize the impact of environmental factors in development of symptoms and disease outcomes. In keeping with recent data, [6,27,28] we observed mutations in all parts of the *FOXP3*-gene, but there was no correlation between the site of mutation and the cellular or clinical phenotype. It is important to note that mutations within FOXP3 resulted in reduced or abolished protein expression in only 4 of 7 patients, while close to normal FOXP3-protein levels were observed in the remaining patients. Confocal immunofluorescence analysis in patient 7 showed cytosolic FOXP3 expression without nuclear localization, as normally observed in healthy controls and other AIE patients with subnormal FOXP3-protein. This patient displays a mutation in the N-terminal part of exon 10 (c.1015 c>g) within the forkhead domain, responsible for the nuclear import of FOXP3-protein. These data are in keeping with recent cell biological analyses, showing that sequences at both the N- and C-terminal parts of the FKH domain are implicated in the nuclear import of FOXP3.[29] In contrast, patient 4 with a missense mutation in the middle part of exon 10 (g.1121 t>g) had low levels of FOXP3 mRNA and protein but a normal nuclear pattern of FOXP3 expression, indicating that this mutation on exon 10 does not interfere with the crucial sequences for nuclear translocation. Bacchetta et al [18] recently suggested that, in IPEX patients, effector T-cell functions might be altered, and they observed a reduced production of IFN-γ and IL-2 upon T-cell receptor stimulation. In our experiments, fresh T-cells from all IPEX patients had impaired IFN-γ production compared to AIE patients, as well as controls. This difference might reflect the intensity of immunosuppression, which is adapted to the activity of disease and was stronger in those IPEX patients who failed to remain in remission. Because all patients were on heavy immunosuppression at the time of blood sampling (no samples of untreated patients were available), we analyzed in parallel cytokine production by $CD4^+$ polyclonal T-cell lines cultured for at least 4 weeks to wash out the immunosuppressive drugs. Cell lines of all IPEX and AIE patients produced much higher amounts (10- to 100-fold) of IFN-γ, IL-13, and IL-17 than polyclonal cell lines from healthy controls. No clear difference was seen between patients with and without mutations of FOXP3. Altogether, these data indicate that there is no intrinsic defect in cytokine production by $CD4^+$ cells from IPEX or non-IPEX AIE patients

and that the impaired cytokine production observed in fresh T-cells is a consequence of external factors, such as heavy immunosuppression. The finding of simultaneously increased Th-1 and Th-2 (and also Th-17) responses is in agreement with the observation of excessive cytokine production by T-cells in naturally occurring scurfy mice.[10] In summary, this single-center series of 11 patients with early-onset AIE points to the critical role of regulatory T-cells in maintaining intestinal homeostasis. Altered FOXP3 expression or function related to genetic defects or defective regulation of FOXP3-protein expression are most often seen in patients with early-onset AIE. However, rare *FOXP3*-independent forms of early-onset AIE exist. *FOXP3*-dependent forms seem to have a more severe clinical course compared to *FOXP3*-independent forms. This clinical series indicates the complexity of AIE and immune regulation and clearly confirms that $FOXP3^{+}$ regulatory T-cells play a key role, but a view of autoimmunity restricted to FOXP3 is incomplete and insufficient and does not explain all aspects of regulatory and effector T-cell interactions.

3.2.5 Acknowledgments

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CHAPTER 4

IMMUNOSUPPRESSIVE DRUGS IN AIE

Severe Dysimmune Cytopenia in Children Treated with Tacrolimus After Organ Transplantation

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Am J Transplant. 2006 May;6(5 Pt 1):1072-6.

Rapamycin Therapy for Severe Autoimmune Enteropathy - Analysis of the Molecular Mechanisms

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Submitted.

4.1 Severe Dysimmune Cytopenia in Children Treated with Tacrolimus After Organ Transplantation

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Abstract

Rare cases of dysimmune phenomena after solid organ transplantation were described in the past. In the present series, we describe six children who developed severe dysimmune anemia or thrombocytopenia while treated with tacrolimus after liver or small bowel transplantation. All patients were off steroids or under low doses alternate day steroid medication when dysimmune cytopenia developed. All patients had positive anti-platelets antibodies and/or Coombs' positive anemia. Therapy was successful in all six patients with a rapid response to corticosteroids in three children, and to anti-CD20 monoclonal antibodies (rituximab) in the three others. The pathogenesis of these rare dysimmune/autoimmune disorders might be related to the interference of tacrolimus with T-cell functions and/or the endogenous control mechanisms of T-lymphocyte activation and down-regulation. Although rare, these complications must be known when discussing protocols of immunosuppression.

4.1.1 Introduction

Tacrolimus is a potent immunosuppressive drug, increasingly used in pediatric transplantation. The introduction of tacrolimus in the early 1990s decreased the rate of acute and chronic rejection after liver transplantation (1), and dramatically improved the outcome after small bowel transplantation (2). The molecular mechanisms of tacrolimus effect are well known: it forms a molecular complex with intracellular FK506-binding protein-12 (FKBP12), thus inhibiting calcineurin, a calcium-dependent serine/threonine protein phosphatase (3). Upon T-cell receptor (TCR) stimulation, calcineurin dephosphorylates various substrates, such as nuclear factors of activated T-cells (NF-AT) or cAMP response element-binding protein (CREB) in T-lymphocytes (4). NF-AT, a family of transcription factors regulating lymphokine gene expression, such as interleukin-2, have been shown to play a prominent role in tacrolimus-induced immunosuppression (5). Depending on the method of evaluation, tacrolimus is 10–50 times more immunosuppressive than cyclosporin (6). Paradoxically, some patients treated with potent immunosuppressive agents after solid organ transplantation develop signs of immune dysregulation, ranging from asymptomatic auto-antibodies (7), to autoimmune hepatitis (8). These observations raise the question of a causal role of the immunosuppressive drugs themselves in a critical perturbation of the control and down-regulation of auto-reactive cells, thereby triggering dys- or autoimmune reactions. We report here six children who developed dysimmune cytopenia after liver or intestinal transplantation, and discuss the role of

tacrolimus in this complication.

4.1.2 Case Reports

Clinical and biological characteristics of the patients are reported in Table 1.
All children, except patient 2, had previously received packed red cells, but no platelet transfusions, during surgery of their primary malformation. The presence of irregular hemagglutinins and Coombs' test were systematically controlled before transplantation and were negative. The first two patients had received a liver transplant with cyclosporin as a primary immunosuppression. Tacrolimus was introduced respectively 6 months and 3 years later because of chronic rejection. Prednisone was suspended 4 and 7.5 years after transplantation. Four children had received a liver and intestinal transplant for short bowel syndrome and end-stage liver disease, with tacrolimus from the first day. Patient 5 who was Rhesus negative and transplanted with organs from a Rhesus-positive donor, received on days 1 and 2 an injection of anti-D immunoglobulins. The absence of anti-Rhesus immunization had been controlled. At the onset of dysimmune disease, these four children were also treated with a low dose of prednisone (0.2 mg/kg or less) on alternate day. At that time, all six children had a trough blood level of tacrolimus between 5 and 10 ng/mL, as measured by an immunoenzymatic assay (EMIT 2000 tacrolimus Dade Behring).
Patients 1 and 2 presented with acute severe thrombocytopenia, mucosal bleeding and a high titer of anti-platelets autoantibodies. From the second year post-transplantation, while treated with tacrolimus for 5 months, patient 1 also suffered from a severe food allergy with angioedema and positive skin tests. Mucosal bleeding started 10 days after a vaccination against influenza. Patient 2 had previously a low platelet count (between 80 and 100 000/mm3), due to a hypersplenism slowly developed from the transplantation, and related to chronic rejection. Anti-platelet antibodies were regularly controlled and negative, until the episode described here. Both children were treated with high-dose prednisone (2 mg/kg), with subsequent tapering. A relapse of thrombocytopenia followed an attempt to completely suspend prednisone in patient 1: a dose of 0.2 mg/kg on alternate day was thus maintained, with a good control on the platelet count together with the food allergy. Patient 2 needed a higher dose of prednisone (0.3 mg/kg every day) to maintain a platelet count over 30 000/mm3. After a mild head trauma he developed an extradural hematoma, and recovered uneventfully. He died 3 years later from septicemia while waiting for re-transplantation for chronic rejection. Patients 3, 4 and 5 developed acute anemia with a positive Coombs' test. Two children were initially treated with i.v. methylprednisolone (300 mg/m^2). In patient 4, the hemoglobin level normalized within 2 weeks. The boy was maintained on a low dose of alternate day prednisone (0.2 mg/kg). During the 3 years of follow-up anemia did not relapse. Patient 3 on the contrary needed daily blood transfusions, underwent an emergency splenectomy, developed severe postsurgical complications and was ultimately treated with

Table 1: Clinical and biological characteristics of the six children at onset of dysimmune disease

		Initial diagnosis	Type of graft	Blood group recipient/ donor	Age at transplantation (years)	Age at tacrolimus (years)	Acute rejection	Delay tacrolimus-cytopenia (years)	Lowest hemoglobin or platelet count	Treatment	Time between onset and first remission[a]
1	F	Biliary atresia	L	A+/A+	1	3	1 month	2	2 000/mm^3	Prednisone	2 months
2	M	PFIC	L	B+/B+	5	8	5 and 9 months	4	3 000/mm^3	Prednisone	1.5 months
3	M	Hirschsprung	L + SB	O+/O+	6	6	0	2	35 g/L	Methylprednisolone, splenectomy, anti-CD20	4 months
4	M	Vascular malformation +NEC	L + SB	A+/A+	4	4	0	2.5	50 g/L	Methylprednisolone	2 weeks
5	M	Hirschsprung	L + SB	A−/A+	6	6	Liver: 1 month	0.5	50 g/L	Anti-CD20, prednisone	1 month
6	M	Hirschsprung	L + SB	AB+/B+	4	4	0	4	10 000/mm^3–50 g/L	Methylprednisolone, anti-CD20	3 weeks

Abbreviations: M, male; F, female; PFIC, progressive familial intrahepatic cholestasis; NEC, necrotizing enterocolitis; L, liver; SB, small bowel; anti-CD20: anti-CD20 monoclonal antibodies.
[a]Remission: platelets > 80 000/mm^3, or hemoglobin > 100 g/l without blood transfusion.

Table 1

four injections of anti-CD20 monoclonal antibodies (9) (rituximab, Mabthera®, Roche, UK, 375 mg/m2, once a week). He recovered slowly, and was maintained on a high dose (1 mg/kg) of prednisone for 1 year. The anemia relapsed when the prednisone dosage was decreased to 0.3 mg/kg daily. The boy received four additional injections of anti-CD20 antibodies; the haptoglobin returned to normal within 1 month. During the following 3 years he was doing well while receiving a low dose of steroids (0.12 mg/kg on alternate day). Patient 5 developed anemia while on a very low dose of prednisone (0.1 mg/kg) for EBV-induced post-transplant lymphoproliferative disorder (PTLD). The PTLD was considered as controlled, as evaluated on the clinical symptoms, the serum level of gammaglobulins, the absence of activated B cells or B-cells clones and the quantitative PCR for EBV. The boy received seven injections of anti-CD20 antibodies. The dosage of prednisone was increased to 1 mg/kg for 2 weeks after the second injection, because of insufficient control of the hemolysis, and tapered rapidly. The boy recovered from both problems within a few weeks, without relapse after 3 years; he still receives 0.1 mg/kg of prednisone on alternate day. In patient 6, autoimmune anemia and thrombocytopenia were diagnosed simultaneously. The boy had developed asthma 6 months earlier. He received high-dose methylprednisolone and eight injections of anti-CD20 antibodies, and responded clinically and biologically in 3 weeks. The hemolytic anemia (hemoglobin 50 g/L) and thrombocytopenia (80 000/mm3) relapsed 1 year after the first episode, 2 months after an episode of adenovirus-induced diarrhoea, on a daily dose of prednisone of 0.15 mg/kg. He responded rapidly to an increased dosage, and the boy received four additional injections of anti-CD20 antibodies. All children underwent a medullar aspiration at the beginning of the diagnostic procedure in order to rule out a malignancy and look for indices of a viral infection. The bone marrow was normal or regenerative in all cases. Viruses were searched in all episodes: PCR was used for CMV, EBV and adenovirus in all patients, HHV6 and HHV7 only in patients 3–6; serology was performed for parvovirus B19 and Mycoplasma pneumoniae. There was no clinical evidence for herpes simplex, measles (all children had been vaccinated previously), varicella or another eruptive viral infection. As mentioned, the only contemporaneous infections were EBV in patient 5, and adenovirus before the relapse in patient 6. Tacrolimus was not stopped by fear of rejection; however, trough blood levels of tacrolimus were lowered to 5 ng/mL. In all children, the auto-antibodies disappeared and remained undetectable upon clinical remission. However they re-appeared in case of relapse. The only significant side effect of anti-CD20 antibodies was a long-term deficiency in immunoglobulins, so that these children were monthly substituted with i.v. injections of immunoglobulins for a mean of 4 months after the last anti-CD20 injection (up to 6 months).

4.1.3 Discussion

In the present series, we report on six children who developed immune cytopenia after solid organ transplantation. Several reasons might explain the occurrence of hematological ab-

normalities in these patients. One possible cause could be via immunization against minor blood groups after blood transfusions. But in this case cytopenia would immediately follow a new transfusion of blood. This possibility is also very unlikely because irregular hemagglutinins were not found before the described onset of hemolytic anemia. None of these children had received platelet transfusions, excluding previous alloimmunization. A different possibility could be that anemia and thrombocytopenia are part of the symptoms of a lymphoproliferative disorder; in PTLD, bone marrow aspirates show most often an infiltration with B-cells, which was not seen in our patients. In addition, all but one patient showed negative EBV-PCR controls. However, true autoimmune manifestations have been reported in association with EBV infection (10). In patient 5, although the lymphoproliferative disorder was considered as controlled at the onset of acute anemia, the role of EBV as a trigger of autoimmunity can be discussed. Other viruses may be responsible for hematological disorders, such as anemia with parvovirus B19 (11). However this is mostly a central defect in erythrogenesis, and not a peripheral destruction of red cells. Mycoplasma pneumoniae can be responsible for autoimmune hemolytic anemia (12), but it was not demonstrated in any of the patients. With regard to infectious agents, it is interesting to observe that viral infections or vaccinations are often found just before the onset or the relapse of an autoimmune disorder, similar to the clinical course in our patients 1 and 6. There is increasing evidence that a subset of patients is prone to develop dysimmune phenomena after organ transplantation. These immune dysregulations can range from asymptomatic formation of auto-antibodies to severe autoimmune diseases, such as hepatitis or hematological abnormalities (7,8,13). The sequence of events—transplantation, immune suppression, development of autoimmune disease most often starting after steroid weaning—is particularly intriguing. It points out to a critical role of T-cell functions in the autoimmune process, because calcineurin-dependent immunosuppressive drugs potently block T-cell activation and effector functions (3). Another paradox is the fact that the treatment of choice of some autoimmune diseases are T-cell blocking agents, such as azathioprine, cyclosporin or tacrolimus (14). Several hypotheses can be discussed to explain the occurrence of dysimmune reactions after transplantation. One possibility is the production of alloantibodies by lymphocytes from the donor, or graft-versus-host disease (GVHD). Several cases of alloimmune hemolytic anemia after kidney transplantation were reported, the antibodies being most often of anti-D (Rhesus) specificity, and sometimes found retrospectively in the donor's serum (15,16). The onset of anemia was usually in the first weeks after transplantation; only one episode was recorded 4 months after surgery. A severe alloimmune thrombocytopenia was demonstrated in three different patients receiving kidney and liver, respectively, from the same donor (17). The production of alloantibodies derived from passenger donor lymphocytes directed against the HPA1a-platelet antigen caused severe thrombocytopenia within the first weeks after transplantation. The long time interval between transplantation and the occurrence of de novo cytopenia in our patients, and to a lesser extent the absence

of other symptoms of GVHD (cutaneous, hepatic or digestive), makes this mechanism rather unlikely in our patients. A different explanation might be found when analyzing the molecular mechanisms and way of administration of the immunosuppressive drugs. Instead of paralyzing the immune system via inhibition of T-cell activation and effector functions, cyclosporin and tacrolimus might paradoxically enhance autoimmunity under particular conditions. Three different scenarios can be discussed: first, there is some evidence that calcineurin-dependent agents impair thymic functions (18). This may allow an interference with the process of negative selection of T-cells within the thymus, resulting in escape and clonal proliferation of potentially auto-reactive T-cells. An indirect argument that thymic dysfunction might be a pathogenetic key, is the fact that these autoimmune phenomena seem to be more frequent in children compared to adults after solid organ transplantation (19). Secondly, these drugs not only block signaling via the TCR, resulting in the expected inhibition of effector functions, but also they might prevent apoptosis occurring after TCR engagement. Induction of apoptosis is a potent mechanism of control, allowing the elimination of activated T-cells upon stimulation. This mechanism is impaired in patients under immunosuppression. When an auto-reactive T-cell is activated, there is a risk of prolonged cell survival and extension causing autoimmunity (20,21). Lastly, the inhibitory effect of calcineurin-dependent immunosuppressive drugs on T-cells is a priori not selective. Therefore, it can be hypothesized that treatment with tacrolimus not only potently blocks activation and functions of CD4 and CD8 cells but also of regulatory $CD4^{-}CD25$ T-cells. Therefore, 'over immunosuppression' might predominantly paralyze this regulatory T-cell population, resulting in some form of de-inhibition. The biological and clinical consequences might be a loss of this down-regulatory arm, normally controlling potentially auto-reactive cells. There is strong experimental evidence that some autoimmune diseases are secondary to T-cell dysregulation, depending on the genetic background, such as autoimmune diabetes in nod mice: transfer of splenic $CD4^{+}$ and $CD8^{+}$ T-cells to nod-scid mice is sufficient to induce the autoimmune disease (22). There are some controversial data indicating that B cells also contribute to the onset of autoimmune reactions (23). These experimental observations uncover that we are beginning to understand the consequences of modifying T-cell functions with immunosuppressive drugs, and the potential to enhance autoimmunity in predisposed individuals. In the present series, dysimmune cytopenia developed only when the children received a low dose of prednisone, and steroid medication was sufficient in three patients to induce remission, as could be expected from autoimmune disorders (24). The further three patients responded favorably to anti-CD20 antibody therapy. For patient 5, high-dose steroids were initially avoided because of the lymphoproliferative disorder. Anti-CD20 antibodies were used successfully in severe autoimmune anemia outside of the context of transplantation (9). Anti-CD20 antibodies induce a depletion of B-cells, thus the secondary disappearance of auto-antibodies. They were used as a salvage drug in patient 3, and as a first-line drug in patients 5 and 6, because of the previous good experience. This

can be discussed, although the side effects are milder than those of steroids. However, their cost, together with the cost of immunoglobulins administered for several months afterwards, has to be taken into consideration. We did not observe signs of dysimmunity in children treated with cyclosporin. A possible explanation is the magnitude of action of the drug, the cyclosporin interfering more weakly with the immune pathways. A switch from tacrolimus to cyclosporin was discussed in all patients. But we feared rejection in the four patients after liver and intestinal transplantation, and it was denied by both other families, because of previous experience with cosmetic side effects. Another potent immunosuppressive drug such as sirolimus was not yet available for patients 3–5 and rapid response to treatment in patient 6 precluded further discussion; his relapse will re-open the debate. In conclusion, we described six patients with severe and potentially life-threatening cytopenia after organ transplantation and discuss the role of tacrolimus. This drug is very efficient against acute and chronic rejection, without the cosmetic side effects of cyclosporin. However, its causal role in inducing the observed dysimmune manifestations is likely. In one child, EBV may also have been involved. Although rare, these complications should be known when using tacrolimus as a first-line immunosuppressant.

4.1.4 References

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4.2 Rapamycin Therapy for Severe Autoimmune Enteropathy - Analysis of the Molecular Mechanisms

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4.2.1 Introduction

Autoimmune enteropathies (AIE) comprise a heterogeneous group of inflammatory conditions of variable severity. Early disease onset, within the first years of life, often reflects a more severe disease evolution.[1] A particularly severe, often lethal form of autoimmune enteropathy of the child was recently described with the discovery of disease causing mutations in the *FOXP3*-gene.[2] *FOXP3*, located on the X-chromosome, is expressed at high levels in $CD4^+CD25^+$ regulatory T-cells. Animal models as well as observations in humans indicate that these cells regulate activation and expansion of effector T-cells.[3,4] There is good experimental evidence that FOXP3 is one major determinant for a T-cell to acquire regulatory functions.[5,6] Genetically determined dysfunctions of FOXP3 cause very severe autoimmune symptoms, particularly of the intestinal tract. In keeping with several experimental, as well as naturally occurring mice models, this observation in humans reflects the crucial role of regulatory T-cells to maintain homeostasis in organs with high antigenic challenge, such as the gastrointestinal tract. This form of AIE associated with several additional autoimmune symptoms is now called IPEX-syndrome (immune dysregulation, polyendocrinopathy, autoimmune enteropathy, X-linked) (OMIM304930). Therapeutic options in IPEX are limited. Most patients have a poor response to immunosuppressive therapy and hematopoietic stem cell transplantation (HSCT) is the only curative option.[7] However, outcome of HSCT is variable. In 2005 we reported for the first time the successful use of the macrolide antibiotic rapamycin (sirolimus, Rapamune®) in 3 patients with IPEX-syndrome.[8] Since then, we treated five more boys with severe IPEX syndrome successfully with rapamycin as longterm maintenance therapy. Compared to calcineurin inhibitors, rapamycin has a markedly better tolerance profile due to less side effects.[9,10] Given the high efficacy of rapamycin in patients with autoimmune enteropathy, we were interested in better understanding the molecular mechanisms of rapamycin compared to tacrolimus in the treatment of autoimmune enteropathy. Rapamune® (rapamycin) does not block T-cell receptor mediated signaling, although it binds to the same FK506-binding protein (FKBP) as tacrolimus. In contrary to tacrolimus, the complex rapamycin FKBP12 blocks a serine/threonine protein kinase, the mammalian

target of rapamycin (mTOR).[11] This mTOR pathway is indispensable for normal protein synthesis and progression in the cell cycle, vital to normal cell functions and survival. In addition to rapamycin-induced inhibition of cells in the late G1 phase, induction of tolerance and anergy by rapamycin were shown in animal models.[12,13] Battaglia et al. demonstrated in murine cells, that rapamycin has a selective effect on regulatory T-cells, showing in vitro expansion of FOXP3$^+$ cells with suppressive functions.[14] In addition, mechanistic studies showed that rapamycin potently blocks cytokine and growth factor induced stimulation, particularly of T-cells.[15] So far, no analyses of the mode of action of rapamycin were performed in patients with defective functions of regulatory T-cells due to mutations in *FOXP3*.

4.2.2 Material and Methods

Patients/Healthy Controls

Five consecutive patients with IPEX, all followed at Necker Enfants Malades Hospital in Paris were included in this study. The diagnosis of autoimmune enteropathy (AIE) was based on the clinical presentation, the typical endoscopic and histological findings, and a genetic analysis confirming a mutation in the *FOXP3*-gene. At inclusion, all boys were between 4 months and 8 years old. All were on immunosuppressive therapy when peripheral blood mononuclear cells (PBMC) were obtained, no blood samples prior to immunosuppressive therapy being available. Upon informed consent, a maximal volume of 10 mL blood was harvested on heparin from all patients and age matched healthy children (n=6); in addition two patients receiving rapamycin-based immunosuppression for small bowel transplantation were included as treatment controls. To set up experimental conditions, initial experiments were performed with blood samples from healthy adult donors, obtained from the "Centre National de Transfusion Sanguine" Paris, France The study is part of a larger investigative project on patients with autoimmune enteropathy. The protocol was approved by our local ethics committee (CPP2 IDF, protocole number AOM08087). All parents gave written consent prior to the inclusion of the individual patients.

Cell Culture

The Jurkat Leukaemic T-cell line A3 (wild type) (ATCC) was cultured during 24, 48 hours and 5 days in a concentration of 5.105 cells in 1 ml of culture medium (RPMI Glutamax® 1640 medium (InvitrogenTM, Cergy Pontoise, France) + 10% FBS) on a 24 multiwell plate. (Falcon®, Becton Dickinson, Le Pont de Claix, France) in a humidified incubator at 37 °C + 5% CO2 in the presence or absence of immunosuppressors tacrolimus or rapamycin (Alexis Biochemicals®) at concentrations of 0.1 -100 ng/ml or sulfazalasine 0.1- 2 mM (Sigma-Aldrich Taufkirchen, Germany).

PBMC were isolated on Ficoll (mononuclear cell separation medium (Eurobio®), density

1,077 +/- 0,001), according to standard protocol. Cells were cultured in RPMI Glutamax® 1640 medium (InvitrogenTM, Cergy Pontoise, France), enriched with 10% decomplemented human serum AB, 1% non essential amino acid,1mM sodium pyruvate, 1mM HEPES buffer, 5×10^{-5} M beta-mercaptoethanol and 50 μg/mL gentamycine (InvitrogenTM). PBMNC were cultured at 37°C in 96-well flat bottomed plates (Falcon®, Becton Dickinson, Le Pont de Claix, France) at the concentration of 2x105 cells/well in a total volume of 200 μL medium. PBMC were stimulated with anti-CD3 (soluble UCHT1 5 μg/mL) combined to anti-CD28 antibodies (0.5 μg/mL) (BD Pharmingen, Le Pont de Claix, France) with or without recombinant-IL-2 (40IU/mL) for up to 72hours (R&D Systems, Lille, France). To obtain polyclonal $CD4^+$T-cell lines, $CD4^+$ cells were negatively selected from PBMC by magnetic cell sorting (MACS®, Miltenyi Biotec, Paris, France), as described below, and stimulated with PHA-L 1 μg/mL (Sigma, St. Quentin Fallavier, France) and IL-2 40 IU/mL and maintained in IL-2 40 IU/mL for a period of 4 to 6 weeks before restimulation by anti-CD3 and anti-CD28 $\pm$ IL-2 for 72 hours. Prior to stimulation, tacrolimus(Alexis Biochemicals®, San Diego, CA, USA) or rapamycin (Alexis Biochemicals®, Lausen, Switzerland) was added at concentrations of 10 and 100 ng/ml.

Flow cytometry

Antibodies directed against CD3, CD4, CD25, (all from BD Biosciences-Pharmingen,Le Pont de Claix, France) were directly coupled to FITC, PE or APC according to manufacturer instructions. Cells were analyzed by flow cytometry (BD LSR).

Cell Seperation and co-culture

$CD4^+CD25^+$ and $CD4^+CD25^-$ T-cells were isolated from PBMC by magnetic cell sorting (MACS) combining two steps: first CD4-negative cells were selected using a cocktail of biotin-conjugated monoclonal antihuman antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ and glycophorin A, and microbeads conjugated with monoclonal anti-biotin antibody (clone Bio3-18E7.2; $CD4^+CD25^+$ regulatory T-cell isolation kit; Miltenyi Biotec, Paris, France) This negative selection step resulted in an approximately 95% pure population of $CD4^+$ cells which was then positively sorted for $CD25^+$ cells, using microbeads conjugated with monoclonal anti-CD25 antibody (clone 2A3). The magnetic separation was performed on one LD column for the negative- and two MS columns for the positive selection process, respectively, according to the manufacturers' instructions (Miltenyi Biotec). The non-retained $CD4^+CD25^-$ and the selected $CD4^+CD25^+$ cells were respectively 85% and 85-90% pure as assessed by flow cytometry. Cells were cultured in RPMI Glutamax® 1640 medium (InvitrogenTM, Cergy Pontoise, France), enriched with 10% decomplemented human serum AB, 1% non essential amino acid, 1mM sodium pyruvate, 1mM HEPES buffer, 5×10^{-5} M beta-mercaptoethanol and 50 μg/mL gentamycine (InvitrogenTM) at 37°C in 96-well flat

bottomed plates (Falcon®, Becton Dickinson, Le Pont de Claix, France). $CD4^+CD25^+$ regulatory T-cells and $CD4^+CD25^-$ effector cells were cultured alone or co-cultured with $CD4^+CD25^+$ regulatory T-cells at a 1:1 effector to regulator cell ratio at the concentration of a total number of 1x105 cells/well in 200 μL medium were stimulated with anti-CD3 (coated UCHT1 5 μg/mL) and anti-CD28 antibodies (soluble 1 μg/mL) (BD Pharmingen, Le Pont de Claix, France) with or without rIL2 (100 IU/mL) (R&D systems), rIL15 (25 ng/mL) (SIGMA) and/or LY (20 mM/mL)(SIGMA) or JAK3 inhibitor (0.168 μM) (Calbiochem).

Cell death assays

Apoptosis was monitored using the annexin V assay with propidium iodide counterstaining (Becton Dickenson, Pont de Claix, France) allowing quantification by flow cytometry (BDLSR; Becton Dickinson). Cells positive for annexin V and negative PI (early apopotic) and annexin V and PI positive were considered as apoptotic.

Proliferation Tests

To evaluate the suppressor activity after 4 days cultures with coated anti-CD3 antibody (5 μg/mL) and anti-CD28 antibody (1 μg/mL) , cells were pulsed with 3H-thymidine for 18 hours and incorporation was measured, on a Wallac 1450 microbeta/ Trilux counter (Perkin Elmer, Courtaboeuf, France) as previously described (Bennet et.al Immunogenetics 2001). Suppressor activity of $CD4^+CD25^+$ cells was determined as the difference between proliferation rates of anti-CD3-stimulated $CD4^+CD25^-$ cells cultured alone or in co-culture with $CD4^+CD25^+$ cells.

Real-Time PCR (RT PCR) analysis of IL-2 mRNA

Total RNA was extracted from fresh PBMCs using Trizol reagent (Invitrogen,Carlsbad, CA) according to the manufacturer's instructions. First strand complementary DNA(cDNA) synthesis was performed using the Omniscript RT Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. IL-2 cDNA levels were quantitated using a Real-Time PCR System (ABIPRISM 7300 – Applied Biosystems) and Platinum SYBR Green qPCR Supermix-UDG (Invitrogen, Carlsbad, 10 CA). Amplification was carried out in a total volume of 20 μl for 40 to 50 cycles with denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. Samples were run in triplicate and their relative expression was determined by normalizing to expression of the housekeeping gene GAPDH. Values obtained, were compared to the level of expression in a normal control sample to calculate the relative fold-change. The following primers were used: IL-2: 5'-CTC ACC AGG ATG CTC ACA TTT AAG-3' and 5'-CCA GTG GTT TGA GTT CTT CTT CTA GAC-3', for GAPDH:5′-CCACATCGCTCAGACACCAT-3′.and 5′GCAACAATATCCACTTTACCAGAGT3′.

ELISA studies

The secretion of various cytokines into the medium of stimulated Jurkat as well as PBMC or purified $CD4^+$ cells, was quantified using the following Elisa kits: IFNγ (R&D SYSTEMS Duoset ref DY285), IL-13 (R&D SYSTEMS Quantikine IL-13 ref D1300), IL-17 (R&D SYSTEMS Duoset ref DY 317),IL-2 (R&D SYSTEMS Quantikine IL-2 D2050), according to the instructions of the manufacturer. Optic density was analysed at 450nm by spectrometry. We calculated the cytokine concentration by regression analysis with the use of a standard curve.

Statistical analyses

Data are presented as means of at least 3 independent experiments. The non parametric Mann Whitney-test was used to compare different groups. P values less than 0.05 were considered statistically significant.

4.2.3 Results

Effect of rapamycin and tacrolimus on T-cell survival and proliferation in IPEX-patients

The effects of rapamycin and tacrolimus on T-cell survival and proliferation were tested in parallel in the Jurkat T cell line, PBMC and purified $CD4^+$ T-cells of healthy controls and IPEX-patients. Neither rapamycin, nor tacrolimus had any toxic or pro-apoptotic effects on Jurkat T-cells.(Fig. 1A) Rapamycin had no effects at 24 and 48h, however, it reduced the spontaneous apoptosis rate at 4d in a dose-dependent manner ($p<0.001$). In keeping, anti-CD3/CD28-stimulated PBMC of healthy donors, as well as IPEX-patients showed no signs of necrosis or apoptosis after rapamycin or tacrolimus treatment beyond baseline stimulation (data not shown). In addition, we tested either drugs' effects on highly purified $CD4^+$ subpopulations: $CD4^+CD25^-$ T-effector cells were separated from $CD4^+CD25^+$ regulatory T-cells by magnetic cell sorting, followed by stimulation for up to 4 days with coated anti-CD3 (5μg/mL) and anti-CD28 (1 μg/mL) antibodies in the presence of rapamycin or tacrolimus. Neither drug enhanced CD3/CD28-induced apoptosis in a significant manner in $CD4^+CD25^-$ T-effector cells (Fig.1B), nor $D4^+CD25^+$regulatory cells (data not shown) On the other side, TCR-induced apoptosis was not reduced by either immunosuppressor, in contrast to the rescue effect of IL-2 or IL-15 (Fig 1C). Given the limited quantity of purified $CD4^+$ cells in IPEX patients, theses apoptosis experiments were only performed in sorted cells of healthy donors. The effect of rapamycin or tacrolimus on T-cell proliferation was tested first *in vitro* (Jurkat T-cell line), combining microscopic cell counts and tritiated thymidine incorporation. Rapamycin had potent anti-mitotic effects on Jurkat T-cells, which were clearly

dose dependent ($p < 0.003$) (Fig.2), while tacrolimus failed to inhibit T-cell growth, even at supra-physiological doses. The anti-proliferative effects of rapamycin were maximal at four days (Fig.2A). In parallel, thymidine incorporation was reduced with rapamycine indicating a strong anti-mitotic effect, while tacrolimus had no effect (data not shown). Subsequently, we tested the effect of rapamycin and tacrolimus on total PBMC or isolated T-effector subsets. When total PBMC or $CD4^+CD25^-$cells were stimulated with 5μg/mL anti-CD3 antibody, rapamycin potently inhibited T-cell proliferation while tacrolimus had a weak inhibitory effect (Fig.2B). Results in PBMC of IPEX-patients were similar with a potent anti-proliferative effect of rapamycin. However, basal proliferation rates (probably due to ongoing immunosuppressive therapy at blood drawl) were markedly lower in cells of IPEX patients compared to cells of healthy donors (Fig 2C).

In co-culture experiments of $CD4^+CD25^-$ and $CD4^+$CD25high cells (50.000:50.000 cells), the regulatory ($CD4^+$CD25high) T-cells potently blocked $CD4^+CD25^-$ effector cell proliferation with over 85 +/- % in healthy individuals (Fig. 3A). The addition of rapamycin potentated the growth inhibitory effect of regulatory $CD4^+$CD25high cells in controls. Tacrolimus had no additional inhibitory effect under these experimental conditions. (Fig.3A) Five patients with IPEX syndrome with absent regulatory T-cells were tested in parallel. In co-culture experiments, $CD4^+$CD25high cells failed to inhibit $CD4^+CD25^-$ cell proliferation (Fig.3A). However, the addition of rapamycin to the co-culture reduced proliferation rates, while tacrolimus was without any significant effect.

Effect of imunosuppressors rapamycin and tacrolimus on IL-2 production

Since the anti-proliferative effect of rapamycin on PBMC could be indirect via the suppression of stimulatory cytokines, we tested the effect of rapamycin and tacrolimus on the expression and production of Interleukin (IL) 2. Transcripts for IL2 in anti-CD3/anti-CD28-stimulated PBMC of healthy controls were analyzed by real time PCR. A dose dependent suppression of the IL2 transcripts was observed after 4 hours of tacrolimus stimulation, while it disappeared after 12h (Fig.4). The effects of rapamycin on IL-2 mRNA expression were neglectable (data not shown). In keeping, Il-2 production and secretion in the supernatants of TCR-stimulated PBMC or $CD4^+CD25^-$ T-effector cells were markedly decreased in the presence of tacrolimus (fig.5A), while, rapamycin had only a minimal effect on PBMC (fig 5B). These experiments were not performed in children with IPEX.

Effect on cytokine production

To analyze the effect on T-effector cell functions of either immunosuppressor, rapamycin or tacrolimus, Th-1, Th-2 and Th-17 responses in CD3/CD28 (+/- IL-2) stimulated PBMC or T-cell lines were analyzed comparing healthy controls to five IPEX patients. The potential

effects of both drugs were tested within the therapeutic range (1-100 ng/ml). Th-1 cytokine production of PBMC of IPEX patients was markedly lower compared to healthy controls. This might be related to ongoing immunosuppressive therapy, since all patients were under tacrolimus (n=5) based immunosuppression. Therefore, we generated $CD4^+$ T-cell lines for each patient and healthy controls to "wash out" the effects of the immunosuppressors. Indeed, T-cell lines of IPEX patients showed in response to CD3/CD28 stimulation a significantly higher Th-1, Th-2 and Th-17 cytokine production compared to healthy controls. This difference was strongest for IFNγ secretion (100-fold increase), while Th-2 and Th-17 cytokine production was approximately 10-fold increased. In all IPEX patients, similar to PBMC of healthy controls, IL-2 did potentiate T-effector responses with a particularly strong effect on Th-1 and Th-2 cytokine production. In T-cells lines, both immunosuppressors blocked potently IFNγ production and secretion in healthy controls and IPEX-patients. The effect of tacrolimus was less in PBMC of healthy individuals and not detectable in IPEX-patients, already under tacrolimus based immunosuppression. In contrast, to the inhibitory effect on Th-1 responses, no significant effect on Th-2 cytokine production was observed neither in controls nor IPEX-patients. Results for PBMC and T-cell lines were comparable, except an absolutely higher cytokine production level in IPEX-patients. Particularly important is the observation of a strong inhibitory effect of rapamycin on IL-17 production of T-cells, while tacrolimus had no or few effects. This anti-IL-17 effect of rapamycin was present in healthy controls, as well as IPEX patients in both, PBMC and T-cell lines.

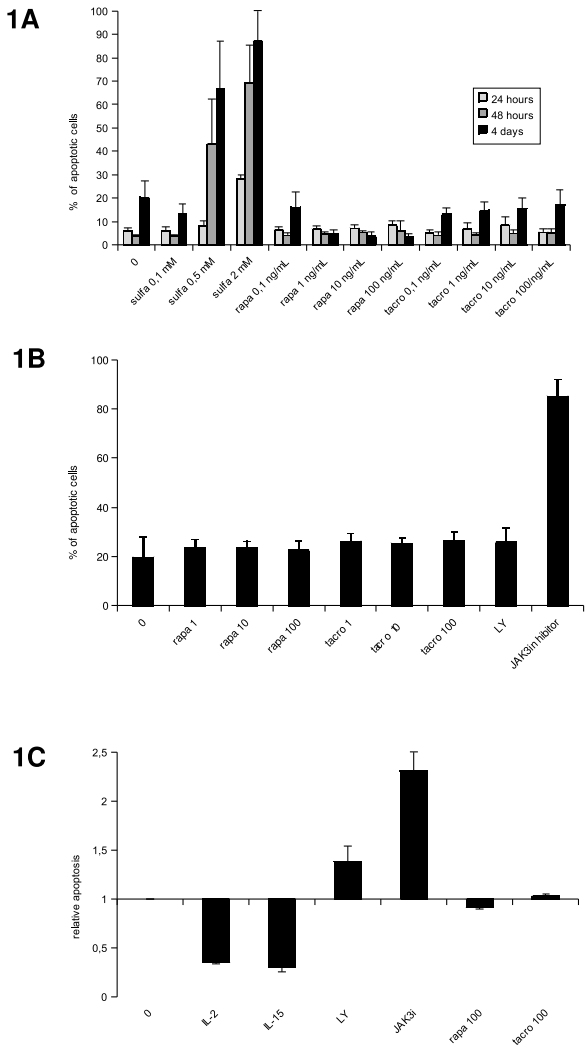

Figure 1. (Figure 1A) Effect of rapamycin and tacrolimus (0,1-100 ng/ml) on the Jurkat T-cell line apoptosis, sulfasalazine (0,1- 2 mM) was used a positive control. Apoptosis was analyzed by the Annexin V/PI method, no pro-apoptotic effects of rapamycin or tacrolimus were observed, even after prolonged stimulation over 4 days, in contrast, rapamycin had an anti-apoptotic effect after prolonged stimulation (*p<0.01) (Figure 1B) Effect of rapamycin and tacrolimus on highly purified $CD4^+CD25^-$ T effector cell subset apoptosis of Healthy Controls. Rapamycin or tacrolimus in parallel to CD3/CD28 stimulation on this cell subsets after 4 days failed to induce apoptosis (Annexin V/ PI method). LY294002 (20μM) and JAK3i (100 μM)-treated cells served as positive apoptosis controls. (Figure 1C) Rescue effect of IL-2 and IL-15 of $CD4^+CD25^-$ T-effector apoptosis after CD3/CD28 stimulation. The immunosuppressors rapamycin or tacrolimus had no effect on purified T effector apoptosis. Data are represented as relative values compared to the apoptosis of stimulated $CD25^-$ cells = value 1.

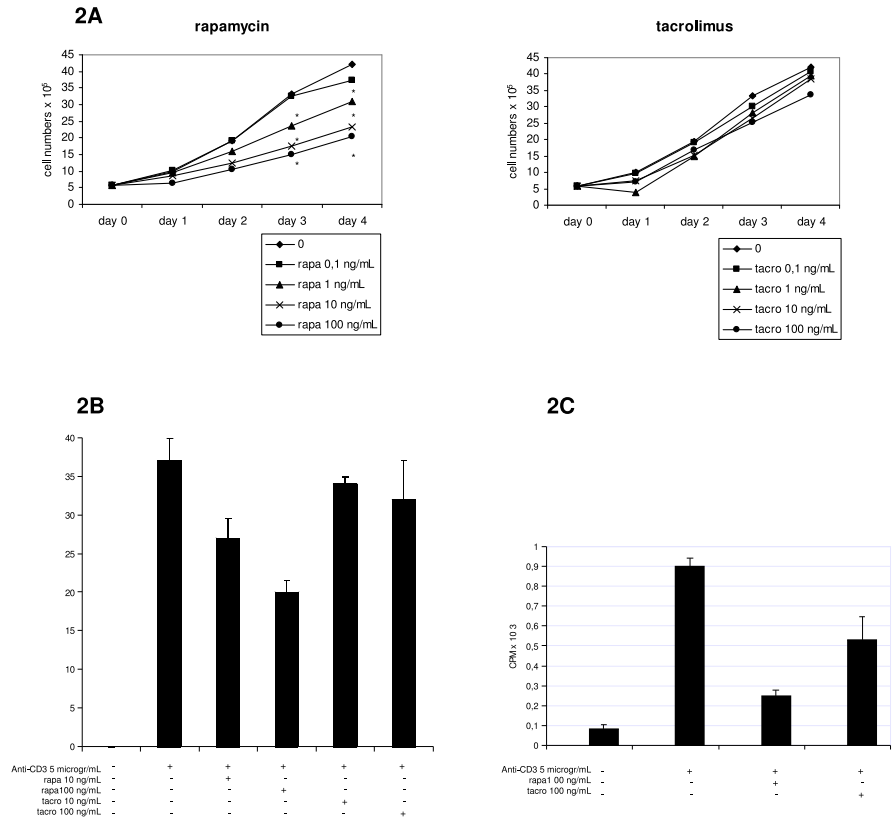

Figure 2. (Figure 2A)Proliferation rate of Jurkat T-cell line measured by absolute cell counts. Marked anti-proliferative effect of rapamycin (0,1-100 ng/ml) on Jurkat T-cells over a 4 day stimulation period, while tacrolimus (0,1-100 ng/ml) was without any significant effect. *$p<0.05$. (Figure 2B) Proliferation rate of PBMC (Healthy Controls) in response to anti-CD3 antibody (5 μg/mL) stimulation for 4 days in the presence or not of the immunosuppressors rapamycin and tacrolimus (3H-thymidine incorporation method). Rapamycin dose-dependently inhibited PBMC proliferation while Tacrolimus showed no effects. (Figure 2C)Proliferation rate of PBMC (IPEX patients) in response to anti-CD3 antibody (5 μg/mL) stimulation for 4 days in the presence or not of the immunosuppressors rapamycin or tacrolimus (3H-thymidine incorporation method). Total 3H-thymidine counts were markedly lower compared to PBMC of healthy controls: rapamycin displayed a strong inhibitory effect on PBMC proliferation while tacrolimus showed only a weak effect.

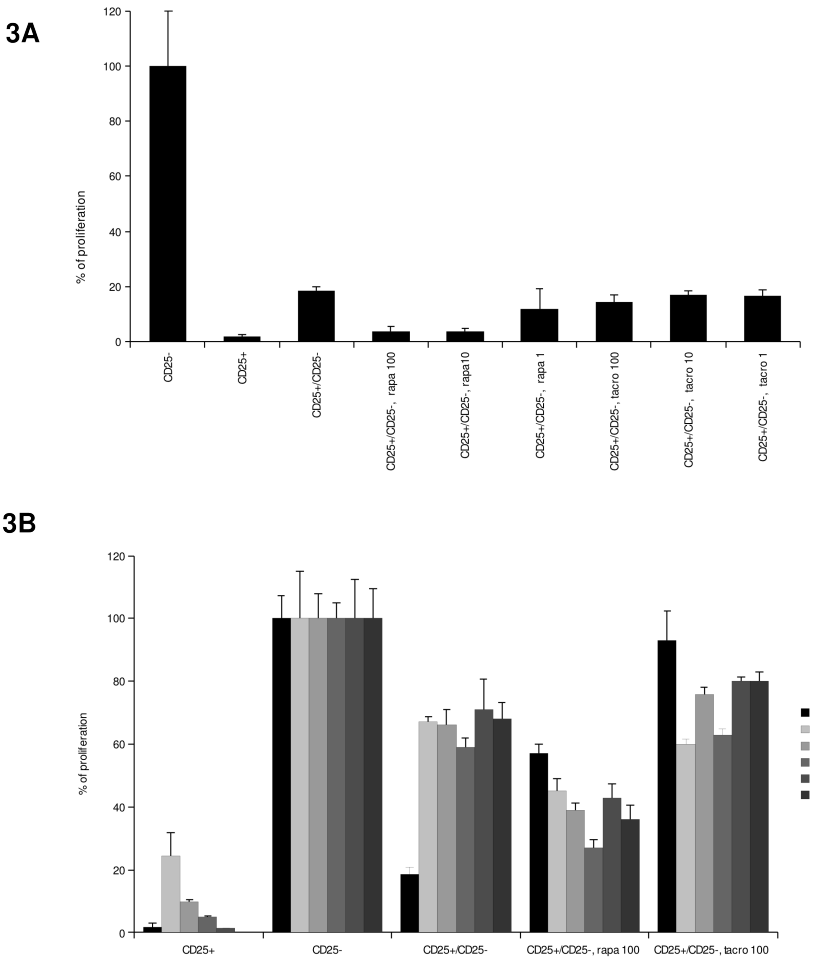

Figure 3. (Figure 3A) Proliferation of Healthy Control $CD4^+CD25^-$ cells and $CD4^+CD25^+$ stimulated for 4 days with anti-CD3 antibody in the concentration of 5 μg/mL cultured separately or in a co-culture system ($CD4^+CD25^-$:$CD4^+CD25^+$ ratio=1:1). The addition of regulatory T-inhibited T-effector proliferation 85±13%. Rapamycin further potentated this growth inhibitory effect of Treg, while tacrolimus had no effect. (Figure 3B) Effect of the immunosuppressor rapamycine or tacrolimus on ($CD4^+CD25^-$: $CD4^+CD25^+$ Co-cultures of IPEX on T-cell proliferation. $CD4^+$CD25high cells of IPEX patients failed to inhibit $CD4^+CD25^-$ T-cell proliferation, however, the addition of rapamycin significantly reduced T-effector proliferation, while tacrolimus was without significant effect in all patients tested.

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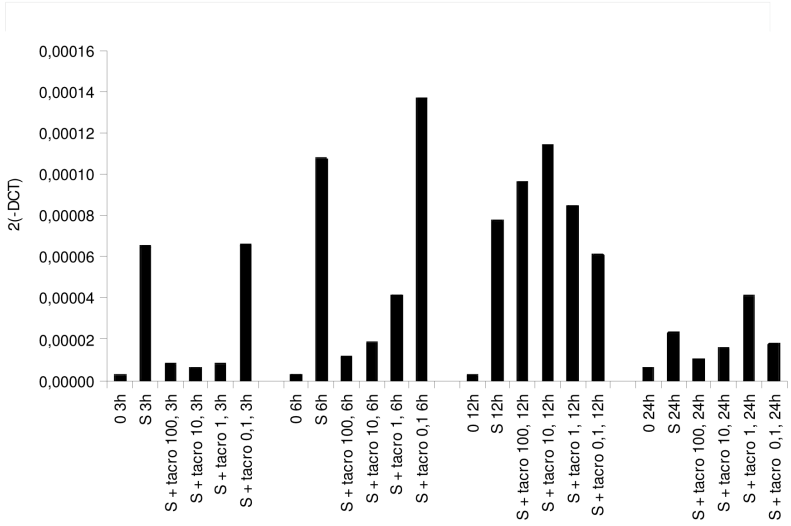
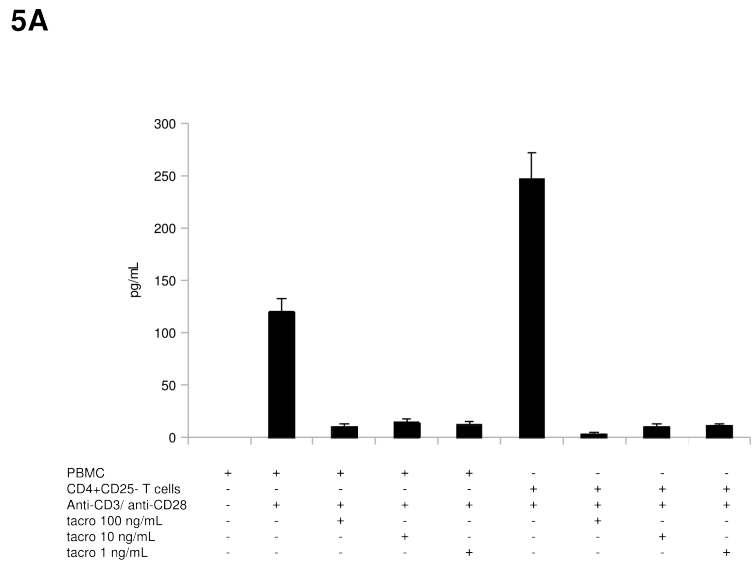


Figure 4. Effect of tacrolimus on CD3/CD28-stimulated PBMC of healthy donors to induce IL-2 transcripts, measured by real time PCR. Tacrolimus potently suppressed anit-CD3/CD28 induced IL-2 transcripts with a maximal effect at 3h.



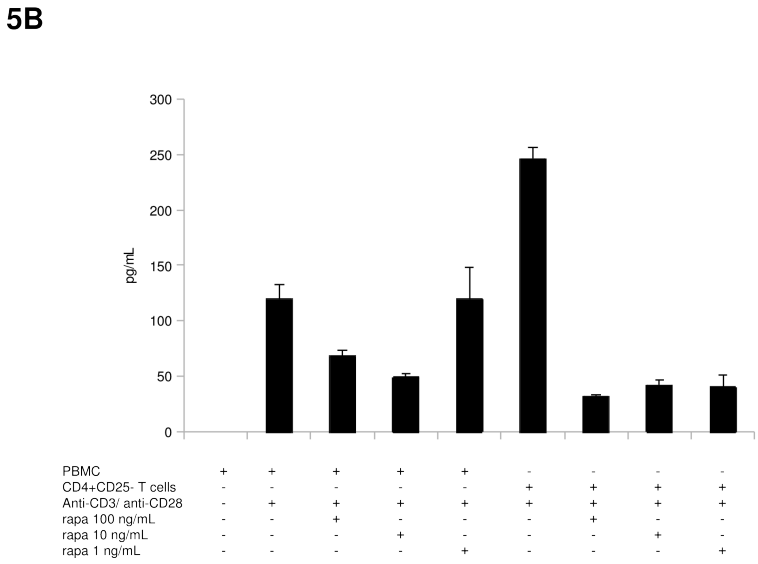


Figure 5. Effect of tacrolimus (A) or rapamycin (B) on interleukin-2 production and secretion by anti-CD3/CD28 stimulated $CD4^{+}CD25^{-}$ effector T-cells of healthy controls measured by ELISA.

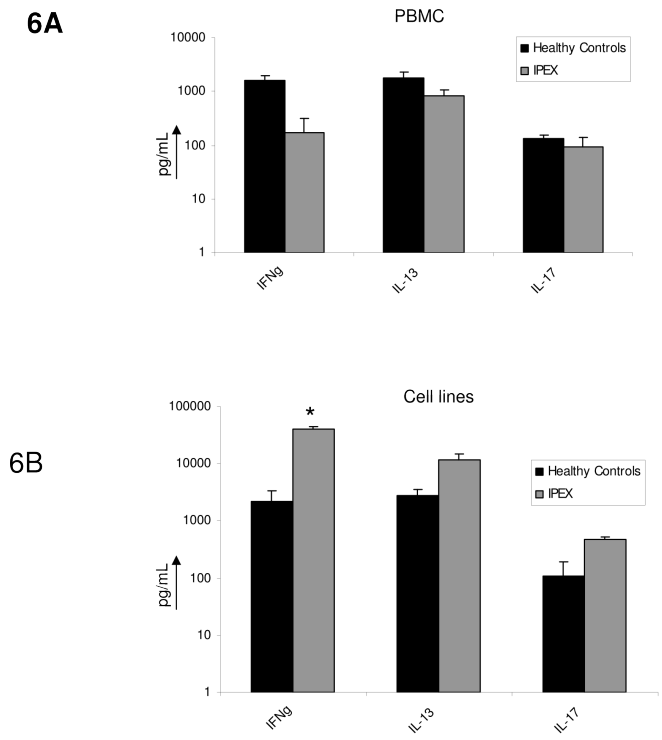
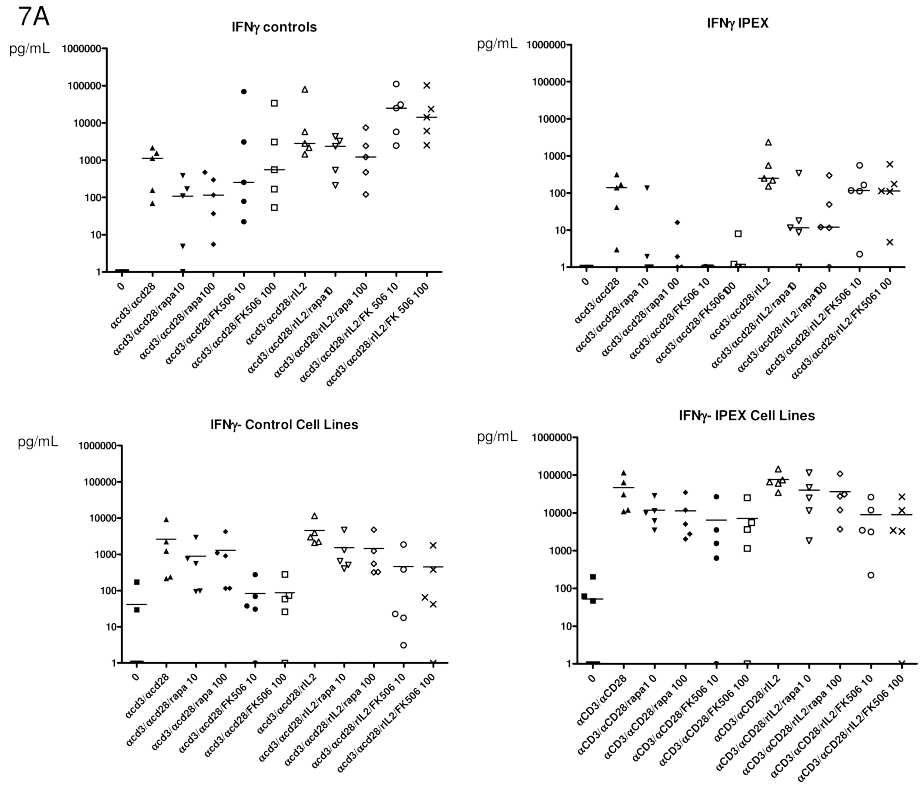


Figure 6. Cytokine production (IFNγ, IL-13, IL-17) by anti-CD3/CD28 -stimulated PBMC of healthy controls compared to PBMC of IPEX (72h stimulation period), showing a markedly reduced overall cytokine production by IPEX patients (A), probably related to the ongoing immunosuppressive therapy. In contrast, T-cell lines derived from PBMC produced significantly 10-100-fold higher cytokine levels in IPEX-patients compared to Healthy controls (B).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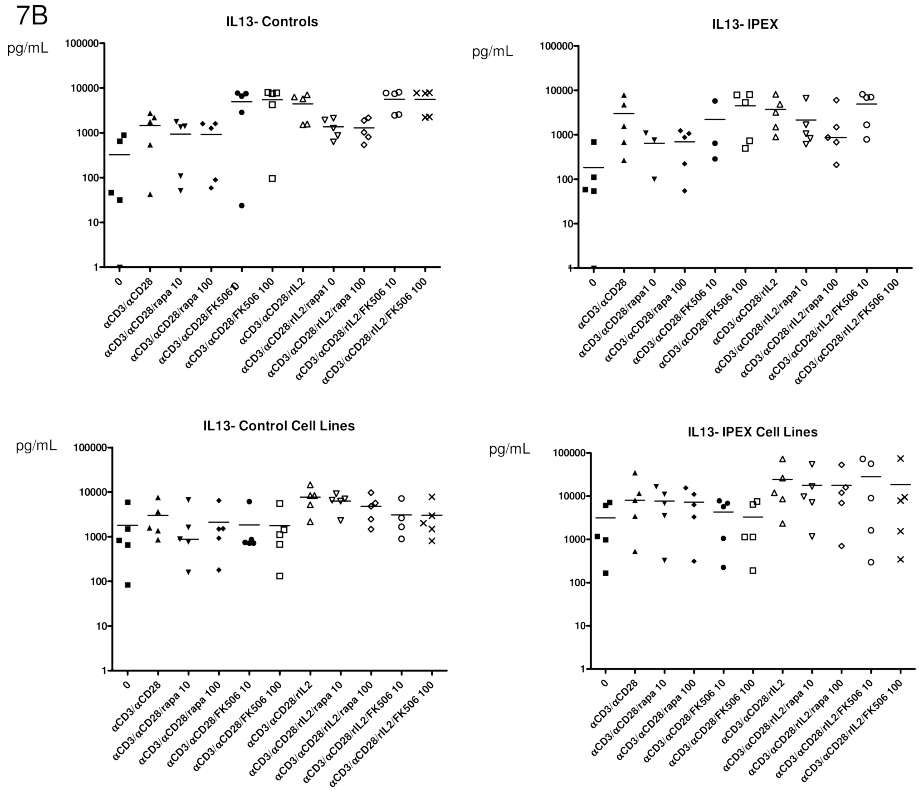
αcd3/αcd28/rapa1 00
αcd3/αcd28/FK506 10
αcd3/αcd28/FK506 00
αcd3/αcd28/rIL2
αcd3/αcd28/rIL2/rapa10
αcd3/αcd28/rIL2/rapa100
αcd3/αcd28/rIL2/FK506 10
αcd3/αcd28/rIL2/FK5061 00
IFNγ- Control Cell Lines
pg/mL
αcd3/αcd28
αcd3/αcd28/rapa 10
αcd3/αcd28/rapa 100
αcd3/αcd28/FK506 10
αcd3/αcd28/FK506 100
αcd3/αcd28/rIL2
αcd3/αcd28/rIL2/rapa 10
αcd3/αcd28/rIL2/rapa 100
αcd3/αcd28/rIL2/FK506 10
αcd3/αcd28/rIL2/FK506 100
IFNγ- IPEX Cell Lines
pg/mL
αCD3/αCD28
αCD3/αCD28/rapa1 0
αCD3/αCD28/rapa 100
αCD3/αCD28/FK506 10
αCD3/αCD28/FK506 100
αCD3/αCD28/rIL2
αCD3/αCD28/rIL2/rapa1 0
αCD3/αCD28/rIL2/rapa 100
αCD3/αCD28/rIL2/FK506 10
αCD3/αCD28/rIL2/FK506 100


7B
IL13- Controls
pg/mL
100000
10000
1000
100
10
1
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αCD3/αCD28
αCD3/αCD28/rapa 10
αCD3/αCD28/rapa 100
αCD3/αCD28/FK506 10
αCD3/αCD28/FK506 100
αCD3/αCD28/rIL2
αCD3/αCD28/rIL2/rapa1 0
αCD3/αCD28/rIL2/rapa 100
αCD3/αCD28/rIL2/FK506 10
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IL13- IPEX
pg/mL
IL13- Control Cell Lines
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IL13- IPEX Cell Lines
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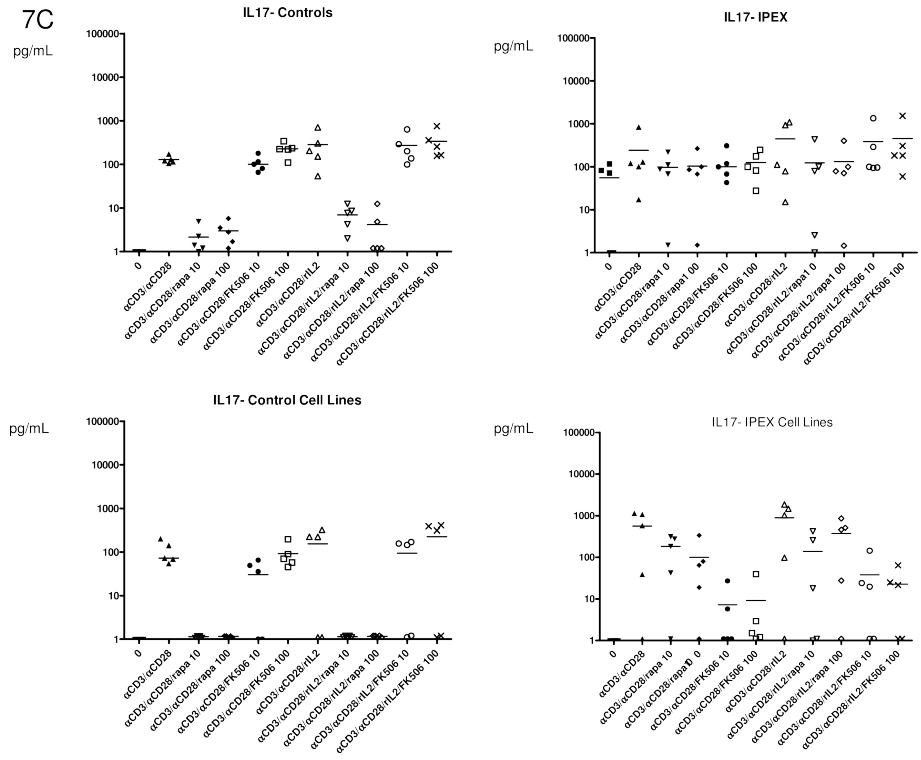

Figure 7. Effect of rapamycin and tacrolimus on anti-CD3/CD28-induced cytokine production in PBMC as well as $CD4^+$ T-cell lines of healthy controls or IPEX patients (3 day stimulation period, ELISA method). Rapamycin had a particular suppressive effect on IL-17 production in both HC and IPEX patients. The effects of rapamycin and tacrolimus were comparable on IL-13 and IFNγ, with the exception that tacrolimus-treated PBMC did not further respond to the suppressive effects of additional tacrolimus. The mean of three independent experiments are shown.

4.2.4 Discussion

The present work aimed to gain insight in the molecular effects of rapamycin (sirolimus)-based therapy in children with autoimmune enteropathy (AIE). We chose to anayse the effects of rapamycin versus tacrolimus on PBMC of healthy controls compared to AIE patients with a mutation in the *FOXP3*-gene. These patients present with a severe and often treatment resistant form of AIE. Given the fact that the causes for autoimmune enteropathies are heterogeneous [16] and regulatory T-cell dysfunction is one of the best characterized molecular defects of autoimmunity [5], we preferred to restrict our analyses on a small but homogenous population of patients with a clearly characterized molecular defect. In all five patients, the genetic defect causes a loss of function and FOXP3-protein was not expressed, leading to a profound dysfunction of regulatory T-cells. This was confirmed in the present study by functional analyses using co-culture experiments. It is important to highlight that boys

with IPEX-syndrome often have a limited life expectancy, particularly if immunosuppressive therapy is not successful and/or if hematopoietic stem cell transplantation is not possible.[1] Therefore, any efforts to improve and stabilize the initial course of the disease are of eminent clinical value. Only few reports looked at the efficacy of different treatment strategies in patients with autoimmune enteropathy. There are some data indicating that calcineurin inhibitors (such as tacrolimus) are efficient to control the inflammatory reaction in patients with autoimmune enteropathy.[17] The successful use of tacrolimus was reported in two boys and one girl, however, genetic data were lacking allowing to further characterized their autoimmune pathology.[17] It is well known, especially from studies in transplantation medicine that tacrolimus has a long list of severe side effects. The most important ones are nephrotoxicity and myelodepression, as well as an increased risk for EBV-induced lymphoproliferation and severe infections [18], compared to a markedly better tolerance of sirolimus. We reported recently the successful use of rapamycin in patients with AIE caused by defective Treg-functions due to mutations in *FOXP3*.[8] Since this initial report, we treated five more IPEX-patients successfully with rapamycin, further confirming the efficacy of this drug in autoimmune diseases on a long-term. Up to now, the molecular mechanisms of successful immunosuppressive therapy (by tacrolimus or rapamycin) in patients with IPEX-syndrome are not elucidated. Therefore, we performed this study based on PBMC of boys with mutations in *FOXP3* and severe IPEX: Our in vitro experiments confirmed that rapamycin has potent anti-proliferative effects on $CD4^+$ cells in healthy controls, as well in children with IPEX-syndrome. In keeping with previous reports this growth inhibition was caused by a strong anti-mitotic effect, without any cytotoxicity or induction of apoptosis.[19] In contrast, we observed a moderate anti-apoptotic effect of rapamycin on long-term stimulation of T-cells (5 days). Strauss L. et al showed a differential effect of rapamycin on Treg- or T-effector cell apoptosis- and proliferation, thereby favoring Treg expansion and survival.[20] We could not confirm this notion in our study. The comparison between IPEX patients (lacking functional T regs) and healthy controls did not show any major differences, however purified $CD4^+CD25^-$ cells of IPEX patients were very limited, therefore, we could not test all experimental conditions. The work of Roncarolo et al. showed recently ex vivo with murine and human T-cells that rapamycin had the capacity to induce and expand regulatory T cells.[14,21] These Tregs, generated in vitro, were functional as confirmed in vivo in experimental animal models.[21] In the present work, we were not able to address this interesting point in our IPEX patients, since they don't express FOXP3 protein, and no induction was measurable even after prolonged rapamycin exposure (data not shown). It is likely that the effect of rapamycin is due to the induction of de novo regulatory T-cells, rather than a direct effect of naturally occurring *FOXP3*-dependent regulatory T cells. This might be also possible in patients with a mutation in *FOXP3* if the process does not require functional FOXP3. Regulatory T-cells require large amounts of IL-2 in order to function in an optimal way and they are dependent on the production of this cytokine by other cellu-

lar sources, mainly effector T-cells. [5,22–25] In a mouse model Pandiyan et al. suggested that competition for IL-2 is a major working mechanism of Treg mediated T-effector cell suppression.[26] The suppressive effect was mediated by trophic cytokine deprivation followed by effector T-cell apoptosis. It is well established that IL-2 stimulates and amplifies effector T-cell responses. Therefore, the effects of immunosuppressive drugs on IL-2 production are of great interest. In the present study, we observed that indeed, both immunosuppressors tacrolimus and rapamycin had the potential to decrease the secretion of IL-2. While the effect of rapamycin was on a posttranscriptional level, tacrolimus completely blocked IL-2 mRNA production and subsequently protein production and secretion. Drug-induced inhibition of IL-2 secretion results in an important immune suppressive effect, independent of the functionality of regulatory T-cells. Scurfy mice, a naturally occurring mouse-model resembling human IPEX-syndrome, develop very high Th-1 and Th-2 cytokine responses.[27] It is interesting to observe that both drugs, rapamycin and tacrolimus were potent suppressor of T-helper responses in controls and patients, as seen in the reduction of cytokine production in response to TCR stimulation. $CD3^{-}CD28$ stimulation induced high Th-1, Th-2 and Th-17 responses in healthy controls and IPEX patients. However, in our IPEX patients, IFNγ levels were surprisingly lower. Recent work suggested that patients with IPEX-syndrome might also have a defect in CD4 effector cells.[28] Yet, another explanation might be the ongoing tacrolimus medication, as was the case for our patients. To address this point and given the fact that no blood samples prior to initiating immunosuppressive therapy were available, we generated CD4 cell lines for each patient and controls enabling an accurate analysis without remnant drug levels or incorporation in T-cells. In fact, Th-1 responses in CD4 T-cell lines were 10-100 fold higher in IPEX patients compared to healthy controls further supporting our hypothesis. It is striking to see that a strong suppressive effect of rapamycin was on IL-17 secretion both in healthy controls and in IPEX patients. This effect was markedly stronger compared to tacrolimus, while tacrolimus was slightly more potent in IFN suppression. Th-2 responses were only minimally if not affected by either immunosuppressor. IL-17 is an important cytokine implicated in the homeostasis of intestinal inflammation.[29,30] It can play a dual role while amplifying inflammatory responses and thereby tissue destruction or by a more protective effect. In IPEX-patients IL-17 levels are highly elevated in the intestinal tissue (personal data) and the fact that rapamycin down-regulated IL-17 might be one specificity of this drug that induces and maintains remission in AIE patients. This effect of rapamycin might also contribute to the balance in the development of T-cells with regulatory versus IL-17 producing cells. Reciprocal differentiation of immunosuppressive $CD4^{+}CD25^{+}FOXP3^{+}$ T-regulatory cells (Tregs) and proinflammatory IL-17-producing cells (Th-17) from naïve CD4 cells was reported to depend largely upon the cytokine environment.[31] Our observation suggests a distinct mode of immunosuppressive action and tolerance induction by rapamycin and tacrolimus in patients with AIE. While rapamycin had a strong antimitotic effect on effector

T-cells, a particular potent reduction of Th-17 responses was observed in healthy controls and IPEX patients. Tacrolimus proved to be more efficacious in the reduction of Th-1 responses in IPEX patients. These first experimental results further underscore the potential of rapamycin in treating patients with AIE.

4.2.5 Acknowledgements

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CHAPTER 5

SUMMARY, DISCUSSION AND FUTURE PERSPECTIVES

5.1 English Summary

Autoimmune enteropathy (AIE) is a rare but severe disease that can present itself early in life. The most severe form presents in the first months of life. To date approximately 150 paediatric patients with AIE have been described in the literature.[1] Mortality among young patients is high with 1-year and 10-year mortality risks of 30% and 50%, respectively. AIE is characterised by the presence of circulating autoantibodies and tissue inflammation caused by autoimmune reaction. In this process, the intestinal mucosa is destroyed. However, the mechanism causing tissue lesions is not well known. In the past it was believed that disease causing antibodies caused the tissue damage. However, nowadays it seems more likely that the occurence of the antibodies is an epiphenomenon and the autoimmune reaction as the primary disease process initiates tissue inflammation and damage, since a kinetic study showed that the auto-antibodies occured after the onset of intestinal lesions.[2] Extensive infiltration of the lamina propria mucosae by T-lymphocytes can produce villous atrophy in both the small intestine and the colon. In addition to affecting the intestinal tract, the disease can lead to other manifestations of autoimmunity such as type 1 diabetes mellitus, autoimmune (hypo- and hyper-) thyreoïditis and autoimmune anemia or thrombocytopenia.[3,4] We now know that regulatory T-cell dysfunction is a key factor in the development of AIE and that some forms of AIE are caused by a mutation in the *FOXP3*-gene.[5,6] Treatment includes immunosuppression or bone marrow transplantation [3] Recently, advances have been made in the understanding of autoimmune enteropathy. However, there are still significant gaps of knowledge regarding the pathogenesis of the disease. A better understanding of the pathogenesis may lead to development of new strategies in diagnosis and treatment that would improve prognosis and quality of life of patients with AIE. New findings would also provide important information on the normal functions of the immune system in the gut. Furthermore, insights on regulatory T-cell function in AIE may be applicable to a larger patient population with other disease states involving dysregulation in Tregs such as diabetes mellitus and conditions following tissue transplantation. This thesis investigated the molecular background of AIE. The study in chapter 2 describes how regulatory T-cell function is influenced by cytokine IL-15. This work suggests that regulatory T-cell function is influenced by environmental factors, which has important implications for our consideration of regulatory T-cell function and it creates new therapeutic opportunities. The studies in chapter 3 describe variations in clinical presentation of AIE. However, a relationship between AIE variation and genotype of the patients was not found. Lastly, the thesis examined the current immunosuppressive treatments in AIE and presents molecular evidence for the first choice of sirolimus for the treatment of AIE.

5.2 Discussion and Future Perspectives

5.2.1 Regulatory T-Cell Function in AIE (Chapter 2)

Research in recent years has lead to a better understanding of the pathogenesis of AIE. It is now believed that natural regulatory T-cell dysfunction plays a key role in the pathogenesis of the disease. Our knowledge on regulatory T-cell function is growing. Previously, it was believed that Tregs could only exert their functions on T-effector cell proliferation and activation, through cell-cell contact-mediated mechanisms. However, recent evidence suggests that soluble factors in the local environment of Treg cells, like cytokines, can play a role in this process by influencing Treg function. [7]

This thesis addressed the question whether cytokine IL-15 influences regulatory T-cell function. From in vitro experiments, we demonstrated a dual effect of IL-15: stimulation of TGF-beta induced regulatory T-cell development and promotion of peripheral CD4+- and CD8+ T-cells resistance to suppression by FOXP3+ regulatory T-cells. We demonstrated that the immunological pathway involved in this effect is the PI3K-AKT pathway. However, the exact mechanism of intervention of the pathway on Treg suppression remains unclear. Recently Pandyian [8] suggested an apoptosis mediated mechanism in a mouse model. However in humans we were not able to reproduce these data. Further research regarding the molecular mechanisms concerning the role of the PI3K-AKT pathway on Treg function, are therefore necessary.

We investigated cytokine IL-15 in particular for a number of reasons. First, there is evidence to suggest that IL-15 plays an important role in the inflammatory response of the intestine. [9] In addition, IL-15 is a key factor in the pathogenesis of celiac disease. Patients with celiac disease are sensitive to gluten exposure, resulting in the disruption of intestinal immunology and causing atrophy of the intestinal mucosa that is very similar to atrophy observed in AIE. [10–13] Second, IL-15 belongs to the same cytokine family as IL-2. [14] Thus, it is expected to share similar immunological pathways to IL-2, which has clear functions in T-cell regulation and pathogenesis of AIE. [15,16] Lastly, IL-15 has not yet been studied in this context. However, IL-15 has its main effect on $CD8^+$ T-cells and AIE is principally a $CD4^+$ T-cell mediated disease [17], making a role for IL-15 in the pathogenesis of AIE less likely. Also, in AIE there is no IL-15 overexpression, as is seen in celiac disease, weakening its possible implication in causing intestinal inflammation and mucosal destruction in AIE. Finally, in the context of AIE it is difficult to explain the meaning of the finding that IL-15 has a positive effect on inducible Treg generation and a negative effect on natural Treg function, since it is thought that dysfunction of the natural cells is the main problem in AIE. One could speculate about inducible Treg cells compensating for non-functional $FOXP3^+$ Tregs. Further investigations need to give more insight in the balance between different Treg sub-populations and their effect on AIE disease course. Our results on cytokine IL-15 await confirmation with *in vivo*

experiments. However, *in vivo* experiments are difficult to achieve in humans. One possibility is to use intestinal biopsies of AIE patients or celiac disease patients, but it is ethically difficult to obtain biopsies from healthy controls that are necessary for comparison. Data from animal studies can not be extrapolated to human disease, as differences in the presentation and pathogenesis of AIE in humans and animals have clearly been demonstrated.

In addition to IL-15, interleukine-17 (IL-17) makes an interesting candidate for further research on cytokine regulation of Treg function. Remarkably, Th-17 development depends on the cytokine TGF-β, which is also linked to regulatory T-cell development and function. Recently, it has been demonstrated that development of murine CD4+ cells towards Th-17 and Tregs is mutually exclusive. In humans there is as yet no direct evidence for antagonistic development of Th-17 cells and Tregs. There is considerable evidence, however, for the importance of IL-17 in the development and progression of inflammatory and autoimmune diseases.[18,19]

Our data on IL-15 support the hypothesis that regulatory T-cell function is not solely dependent on cell-cell contact with T effector cells , but can be influenced by external factors in the immunological environment like cytokines. Data from other research areas covering oral tolerance and infectious diseases show that other external factors like for example food components or bacteria can also have an effect on Treg function. The intestinal mucosa is an inferface with continuous exposure to these foreign antigens. Research in the field of oral tolerance shows for example, that oral stimulation with ovalbumin (an allergenic antigen) leads to the development of oral tolerance through FOXP3+ regulatory T-cells-dependent mechanisms. Mucida et al. recently suggested that the T-cells may not be of thymic origin. [20] Instead regulatory T-cells may be derived by means of mucosal de novo generation. This is supported by findings that genetically engineered RAG -/-mice, which completely lack both B- and T-cells, can gain the ability to develop oral tolerance when repopulated with monoclonal B- and non-regulatory T-cells.[21] These findings indicate an important role of regulatory T-cells within the intestinal mucosa. In addition, Karlsson and collegues showed that tolerance is associated with the appearance of circulating CD4+CD25$^+$ regulatory T-cells. These regulatory T-cells are capable of suppressing the effector T-cells that were generated within a week after reintroduction of cow's milk. Depletion of these CD25$^+$ T-cells from peripheral blood mononuclear cell culture (PBMC) of tolerant children produced a fivefold increase in in vitro proliferation against β-lactoglobulin, another allergenic protein used in allergy models. Specific IgE against cow milk protein was observed in some IPEX patients at very high titers, especially in the IPEX population with very severe immunoallergic reactions. Investigations of oral tolerance in this population could give possible new directions on the role of Tregs in oral tolerance.

The fact that the GI and skin diseases are prominent in IPEX syndrome points to an important role for Tregs in the immune response to bacterial antigens. A recent observation is that CD4+CD25$^+$ Tregs also express receptors of the innate immune system such as Toll-like

receptors 2, 4, 5 and 8.[22–26] enabling them to sense bacterial products. For example, activation of Toll-like receptor 4 on purified murine bacterial CD4+CD25$^+$ T-cells by bacterial lipopolysaccharide increased the suppressor efficiency of these cells [24], whereas activation of Toll-like receptor 2 decreased the suppressive activity.[23] Further reseach is neccesary to better identify the role of these receptors on Treg and their influence on the disease course in AIE. Additional evidence that regulatory T cells adapt to the outside environment comes from transplantation medicine. In this field Tregs play a role in maintaining graft tolerance by balancing their response through mechanisms of antigen specificity. As is true for all other T-cells, Treg suppression is dependent on TCR activation.[27] Recent studies have shown that antigen-specific TCR activation on CD4+CD25- cells can transform these cells to Treg cells. These antigen-specific Tregs have been proven successful in preventing solid organ rejection. Their efficacy in GVHD has also been demonstrated.[28,29] Antigen specific Tregs are superior to non-specific Tregs, which deliver non-specific immune regulation. As a result, a more measured tolerance model is achieved; the graft is protected without over-suppressing the immune system, reducing the risk of patients developing infections or cancers. However, tailoring of the application of these cells for clinical transplantation use is still needed.
Our data demonstrating the effect of cytokine IL-15 on Treg function as well as the aforementioned studies giving more insight in the role of Tregs at these important self/environment interfaces contribute to our understanding of Treg functioning. Based upon these data, we consider Tregs capable of adapting their function to the tissue environment in which they find themselves. This may provide opportunities for new therapeutic interventions. Since Treg dysfunction is considered the main defect in AIE, our belief is that the AIE patients in the future will benefit from these data.

5.2.2 Heterogeneity in AIE (Chapter 3)

IPEX syndrome or*FOXP3*-dependent AIE

The first study in this chapter describes a new phenotype of IPEX syndrome, the *FOXP3*-dependent form of AIE. In this phenotype the enteropathy is combined with severe immunoallergic manifestations, instead of the typical endocrinopathy and skin manifestations thus far described. Based on this study we conclude that IPEX syndrome should be considered in very severe forms of allergy when common treatment strategies are non successful. The patients described show a new mutational defect in the *FOXP3*-gene. Therefore we hypothesized that their phenotype expression could be specific for their mutation. In the second study of this chapter we tested this hypothesis. This second study demonstrates that regulatory T-cell function is impaired in all AIE patients with the *FOXP3*-mutation. However, the extent to which Treg function is impaired differs between individual patients. Yet neither linkage to specific mutations nor to one of the two specific phenotypes of IPEX syndrome

has been identified. Therefore, we conclude that no genotype-phenotype correlation can be made based on our results. This is consistent with the recent observation of Gambineri et.al. [30] If the phenotype of IPEX syndrome is not solely dependent on genetic factors, then it is probable that environmental factors play a role. The fact that two patients with the same *FOXP3* mutation have different clinical phenotypes (chapter 3.2) is in support of this hypothesis. Furthermore, the new concept of T-helper cell plasticity [31] supports the hypothesis that the environmental situation of T-effector cells, influences their cytokine profile. In the light of this concept when patients have non functional Tregs, the dominating cytokine profile due to environmental impact, decides the expression of symptoms. Another interesting observation of this thesis is that some IPEX patients have a *FOXP3*-gene mutation, but express near normal FOXP3 protein levels. Nevertheless, these patients have severely affected regulatory T- cell function. Confocal immunofluorescence analysis in one of those patients showed normal quantity of cytosolic FOXP3, but no nuclear localization. This first study in man is consistent with studies in mice demonstrating that FOXP3 protein outside the nucleus is non functional.

FOXP3-independent AIE

In forms of AIE in which the *FOXP3*-gene is intact, two remarkable results were noticed. First, there are patients with a non-mutated *FOXP3*-gene but expression of lower levels of FOXP3 protein. This suggests that there are factors that can regulate *FOXP3*-gene function. Direct regulation could be possible through modified promotor function, upstream regions or regulatory genes. At least 300 regulatory genes have been identified so far.[32] However, none have been directly linked with the development of AIE. Indirect regulation by environmental factors is also possible. These factors have been shown to modulate other genes, which are implicated in autoimmune diseases such as the autoimmune regulator gene.[33,34]In mice there is evidence for regulation through different immunological pathways like the PI-3 kinase Akt-mTOR pathway. This is a very interesting finding since immunosuppressor rapamycin influences this pathway and is currently giving good clinical results in the treatment of AIE. Studies regarding candidate genes or pathways regulating FOXP3 can contribute to our understanding of regulatory T-cell function and may also give new therapeutic options for AIE. Second, there are patients with AIE without the *FOXP3* mutation and without regulatory T-cell dysfunction. This finding shows that development of AIE is not necessarily always related to problems with regulatory T-cells. Thus, other mechanisms of pathogenesis for AIE must exist.

This chapter demonstrates that autoimmune enteropathy is a heterogeneous disease. Based on genetic background, clinical presentation and immunological characteristics, we suggest distinguishing three types of AIE. [3,13] These forms are: 1) a systemic X-linked variant that is FOXP3-dependent, called Immune dysregulation, Polyendocrinopathy, autoimmune Entero-

pathy X-linked (IPEX)- syndrome, 2) systemic *FOXP3*-independent forms and 3) gastrointestinal AIE.
The clinical phenotype between *FOXP3*-dependent and -independent forms can be overlapping and equally severe. However, despite similar presentation at onset of disease, disease course seems to be milder and prognosis is better for patients with *FOXP3*-independent form of AIE. Currently, diagnostic tests measuring FOXP3-protein levels or Treg functions as disease markers are not fully validated. Furthermore, based on our data, their usefulness in identifying the less severe forms of AIE is questionable. However, IgE levels in our study seem to correspond quite well with severity of disease. Patients with *FOXP3*-dependent AIE showed much higher IgE levels than those with *FOXP3*-independent AIE and milder forms of the disease. Therefore, we suggest using IgE as a marker for severity of AIE.

5.2.3 Immunosuppressive Drugs in AIE (Chapter 4)

Severe forms of AIE are currently treated with T-cell mediated immunosuppressors. To date the best clinical results were obtained with tacrolimus. Downside is that tacrolimus has some serious adverse effects such as kidney toxicity and the possibility of autoimmunity. The first study in chapter 4 describes a number of transplanted patients who developed an allo-immune cytopenia under tacrolimus and discussed what is currently published about the role of tacrolimus in the development of this hematological autoimmune disorder. There are three hypotheses to explain the development of cytopenia under tacrolimus. First, the drug affects the function of the thymus. Second, the drug has an effect on cell death of activated T-cells. Third, the agent seems to blocks regulatory T-cell function next to effector T-cell function, causing immunological imbalance. These hypotheses should be investigated further in an experimental setting. Our data from *in-vitro*- experiments performed on cells of AIE patients have not shown evidence for an effect on apoptosis or Tregs/T-effector cells functions. Because of the unresponsiveness of certain patients to tacrolimus treatment and the severe adverse effects of the drug, we gained experience with another immunosuppressor, namely sirolimus. [35] The first clinical results appear to be positive. In chapter 4.2 we compare tacrolimus and sirolimus in treatment for AIE. This study supports the use of sirolimus for the treatment of AIE. Sirolimus suppresses the cytokine production of T-effector cells to the same extent as tacrolimus. Both drugs had a particular effect on Th-1 cytokine interferon γ. In addition, sirolimus has a clear inhibitory effect on the proliferation of effector T-cells, which tacrolimus lacks. However, a better understanding of the mechanisms of these drugs in AIE is important for obtaining specific approaches for treatment of different forms of AIE. Improved pharmacological approaches are needed because hematopoetic stem cell transplantation results are not optimal in all patients. Further research could focus on the impact of the drugs on the orientation of the immune response or the induction of regulatory T-cells. Sirolimus has been shown to effectively induce regulatory T-cells. [36] Since both

immunosuppressive drugs exert a particular effect on Th-1 cytokine IFN-γ, new agents that also control Th-2 cytokine production is desired. This is especially important for IPEX patients in whom immune enteropathy is seen in combination with allergic reactions in which Th-2 cells play an important role. Initially these studies would be conducted in in vitro models. However in vivo studies are also required because they would give more certainty about the desired effect. In IPEX syndrome patients, the effect and biological activity of sirolimus can be analysed in the intestinal mucosa ex-vivo by analysing the phosphorylation of the mTOR (mammalian target of rapamycin)-targets p70 ribosomal protein s6 kinase and EIF4EBP (Eukaryotic translation initiation factor 4E-binding protein) within the intestinal tissue before and under sirolimus treatment. In parallel, effects on T-cells can be analysed in the same manner in patients under treatment. Besides immunosuppressive therapy, a search for curative treatments for AIE is necessary. Hematopoietic stem cell transplantation (HSCT) is still the only possibility of cure. The first attempts with myeloablative conditioning had only temporary success. All patients died in the first year after transplantation. However, new approaches using other techniques than myeloablation have greatly improved the outcome.[37–39] These HSCT transplants are less toxic and remain stable over a long period. Seven patients with a follow-up of up to 4 years after transplantation have been reported. [1] When hematopoietic stem cell transplantation is performed early, irreversible damage to the pancreas or the thyroid by autoantibodies can be prevented. [1] Extensive research is on-going regarding possibilities for immunotherapy with regulatory T-cells. Gene modifications in mice have made ectopic *FOXP3*-gene expression possible by reprogramming effector T-cells. [40] The generated cells, exhibit many properties of conventional regulatory T-cells. Two studies describe a similar process in human cells. To date, however, genetically modified regulatory T-cells showing *FOXP3*-gene expression in human cells do not show suppressive abilities.[40]

5.3 Final Word

In conclusion, the studies in this thesis contribute to understanding the pathogenesis of AIE. Still there are many steps to undertake in unraveling and ultimately cure the disease.

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CHAPTER 6

NEDERLANDSE SAMENVATTING

Gebaseerd op:

Moes ND, Ruemmele FM, Rings EH. Auto-immuun enteropathie bij kinderen. Ned Tijdschr Geneeskd. 2011;155:A3246. Dutch. PMID: 21527058

6.1 Inleiding

Auto-immuun enteropathie (AIE) is een zeldzaam ziektebeeld dat zich meestal in de eerste levensmaanden presenteert met een ernstige diarree, die niet reageert op elementaire voeding. Tot nu toe zijn in de literatuur ongeveer 150 patiënten met AIE beschreven.[1] Het is een ernstige aandoening met een mortaliteitsrisico van 30% vóór het einde van het 1e levensjaar en van 50% vóór het einde van het 10e levensjaar. Kenmerkend voor de aandoening zijn circulerende autoantistoffen en inflammatie van de darm door een autoimmuun reactie, die verwoesting van de darmwand veroorzaken. Er treedt villusatrofie op met uitgebreide infiltratie van de lamina propria mucosae door T-lymfocyten. Zowel de dunne darm als het colon kunnen zijn aangedaan. Naast het darmlijden kunnen bij AIE ook andere uitingen van auto-immuniteit bestaan, zoals diabetes mellitus type 1, auto-immuun (hypo- en hyper) thyreoïdie en auto-immuun anemie of trombocytopenie.[2,3] Bij AIE blijkt sprake te zijn van een defect van het verworven immuunsysteem, waaraan het niet-functioneren van regulerende T-cellen ten grondslag ligt. Bij een deel van de patiënten wordt dit defect veroorzaakt door mutaties in het *FOXP3*-gen op het X-chromosoom. Behandeling geschiedt door middel van immuunsuppressie of beenmergtransplantatie.

Er is in de afgelopen jaren vooruitgang geboekt in de kennis van autoimmuun enteropathie. Toch zijn er nog veel onduidelijkheden omtrent de pathogenese van het ziektebeeld. Het vergroten van kennis over de pathogenese van het ziektebeeld kan worden gebruikt voor het ontwikkelen van nieuwe strategieën in de diagnostiek en behandeling van patiënten met AIE. Tevens kan het belangrijke informatie geven over de normale werking van het immunologische systeem in de darm.

In de studies van dit proefschrift is de moleculaire achtergrond van AIE nader onderzocht.

6.2 Immunologische achtergrond - regulerende T-cel functie in AIE

In het ontstaan van AIE speelt dysfunctie van regulerende T-cellen een belangrijke rol. Regulerende T-cellen worden in de thymus gemaakt onder invloed van het *FOXP3*-gen.[4] Regulerende T-cellen remmen de proliferatie van effector T-cellen en de productie van cytokines. Het precieze werkingsmechanisme hierachter is nog onbekend.[5] Verschillende onderzoeksgroepen hebben aangetoond dat cel-cel contact hierbij belangrijk is. Een recente theorie is dat de regulerende T-cellen groeifactoren wegvangen, die belangrijk zijn voor de overleving van effector T-cellen (bijvoorbeeld IL-2), waardoor ze apoptose van de effector T-cellen induceren.[6] Ook komt er steeds meer bewijs voor minder directe beïnvloeding door tussenkomst van in het cytoplasma opgeloste factoren, bijvoorbeeld cytokines. Er zijn een aantal artikelen gepubliceerd die hierin een rol wegleggen voor cytokines TGF-beta en IL-

10.[7,8] In hoofdstuk 2 wordt de rol van een ander cytokine op de regulerende T-cel functie onderzocht, namelijk: IL-15. De keus voor dit cytokine is drieledig: Allereerst blijkt dat dit cytokine een belangrijke rol speelt bij het ontstaan van inflammatie van de darm.[9] Tevens speelt IL-15 een centrale rol in de pathogenese van coeliakie, een ziekte waarbij gluten-overgevoeligheid voor een verstoring van de darmimmunologie zorgt en een atrofie van de darmmucosa veroorzaakt die veel lijkt op de atrofie die ontstaat bij AIE.[2,10–12] Bovendien behoort dit cytokine tot dezelfde familie als IL-2, waardoor de werking deels op dezelfde immunologische cascades berust.[13] Het effect van IL-2 en zijn receptor op regulerende T-cel functie en daarmee de rol in de pathogenese van AIE zijn aangetoond.[14,15] IL-15 is tot op heden in deze context niet onderzocht. In hoofdstuk 2 wordt door middel van in vitro experimenten een tweeledig effect aangetoond van IL-15 op regulerende T-cel functie. Aan de ene kant stimuleert het cytokine de ontwikkeling van nieuwe regulerende T-cellen. Aan de andere kant zorgt het cytokine ervoor dat perifere $CD4^+$ en $CD8^+$ T-cellen resistent werden voor onderdrukking van de regulerende T-cellen. De resultaten in dit hoofdstuk ondersteunen de hypothese dat regulerende T-cel functie door omgevingsfactoren beïnvloed kan worden. Dit schept nieuwe aangrijpingsmogelijkheden voor therapeutische interventie. Verder suggereert dit dat de cellen betrokken kunnen zijn bij immunologische reacties gericht tegen lichaamsvreemde antigenen. Dit is interessant in het kader van bijvoorbeeld orale tolerantie. De eerste studies die hierin een rol voor regulerende cellen wegleggen zijn verschenen. Kinderen met AIE zijn extra gevoelig voor het ontwikkelen van voedselallergie en daarom een geschikte patiëntencategorie om door middel van vervolgonderzoek dit verband van regulerende T-cellen en het ontstaan van tolerantie te versterken. Een ander gebied dat nu onderzocht kan worden is de oorzaak voor de verhoogde infectiegevoeligheid van IPEX (Immune dysregulation Polyendocrinopathy autoimmune Enteropathy X-linked) patiënten. Wordt dit enkel veroorzaakt door immuunsuppressieve behandelingen die zij ondergaan of is het intrinsiek aan het ziektebeeld, waarbij regulerende T-cellen de lichaamsvreemde infectiekiemen opmerken door middel van bepaalde receptoren. Aanwijzingen voor het opmerken van lichaamsvreemde infectiekiemen door regulerende T-cellen zijn gevonden in de aanwezigheid van zogenoemde toll-like receptoren (receptoren die een rol spelen in het naive immuunsysteem en reageren op bepaalde bacteriële producten) op de celmembraan van deze cellen.

6.3 Verschillende fenotypen en genotypen van AIE

Het syndroom gekenmerkt door immuundysregulatie, polyendocrinopathie, auto-immuun enteropathie, X-gebonden (IPEX-syndroom) is de ernstigste vorm van AIE en is tevens de best gekarakteriseerde vorm van AIE. Het klassieke beeld van het IPEX-syndroom is een combinatie van enteropathie, diabetes mellitus en hematologische problemen. Bij 75% van de patiënten gaat het om deze trias (zie de tabel in de introductie van dit proefschrift). In

de eerste studie van hoofdstuk 3 (sectie 3.1) wordt een nieuwe variant van het syndroom beschreven, waarbij de enteropathie samengaat met immuungerelateerde allergische reacties, maar waarbij er geen sprake is van diabetes.[16] Deze combinatie blijkt uiteindelijk bij 25% van de patiënten te worden aangetroffen (zie de tabel in de introductie van dit proefschrift).
Sinds een aantal jaren is bekend dat mutaties in het *FOXP3*-gen het IPEX-syndroom veroorzaken. Dit is ontdekt doordat jongens met het IPEX-syndroom duidelijke klinische overeenkomsten vertonen met zogenaamde *Foxp3*-knockout-scurfy-muizen. De mutaties in het *Foxp3*-gen bij deze muizen veroorzaken een letaal auto-immuunsyndroom met massieve infiltratie van lever, longen, pancreas, huid en darmen door ontstekingscellen, dat een grote overeenkomst vertoont met het IPEX-syndroom bij kinderen.[17,18] Het humane *FOXP3*-gen is gelegen op het X-chromosoom. Het IPEX-syndroom is daarmee een aandoening die alleen jongens treft. Het gen bestaat uit 11 exonen.[17,18] In al deze exonen zijn mutaties gevonden die leiden tot het ontstaan van AIE (figuur 1). In de tweede studie (sectie 3.2) in hoofdstuk 3, is geprobeerd mutaties in verschillende exonen te correleren met de verschillende fenotypen van het IPEX-syndroom, maar dat is tot op heden niet gelukt. Dit resultaat is in overeenstemming met de recente observatie van van Gambineri et al.[19] Verklaringen hiervoor kunnen gezocht worden in omgevingsfactoren, ziekte modulerende genen of infecties. In de discussie worden deze verklaringen nader besproken. Daarnaast gaat de tweede studie van dit hoofdstuk (sectie 3.2) in op ernstige, vroege vormen van AIE die niet lijken te worden veroorzaakt door een mutatie in het *FOXP3*-gen. De genetische achtergrond van deze autoimmune enteropathieën is op dit moment nog niet verklaard. Er wordt onderzoek gedaan naar het IL-2-receptor-gen.[8] Patiënten met IL-2-receptordeficiënties hebben namelijk eenzelfde klinische presentatie als patiënten met IPEX-syndroom. Daarnaast wordt onderzoek gedaan naar genen die het *FOXP3*-gen reguleren. Daarvan zijn er tot op heden ten minste 300 aangetoond.[20] Echter, voor geen van allen is tot nu toe een direct verband met het ontstaan van AIE bewezen. De *FOXP3*-afhankelijke vormen van AIE lijken ernstiger te verlopen dan de *FOXP3*-onafhankelijke vormen.
Op basis van genetische achtergrond, klinische presentatie en immunologische kenmerken onderscheiden we hedendaags 3 typen AIE.[2,21]
1. Een systemische X-linked en FOXP3-afhankelijke variant: Immune dysregulation, Polyendocrinopathy, autoimmune Enteropathy X-linked (IPEX)- syndrome.
2. Systemische *FOXP3*-onafhankelijke vormen.
3. Gastrointestinale AIE.

Samenvattend komt in hoofdstuk 3 naar voren dat AIE een veel heterogener ziektebeeld is dan dat tot nu toe werd aangenomen. De klinische presentatie van patiënten kan verschillen en de genetische achtergrond is niet eenduidig. Dit betekent dat bij een veel groter scala aan patiënten aan de ziekte gedacht kan worden. Met de huidige diagnostische testen kan het

ziektebeeld goed onderscheiden worden van bepaalde andere inflammatoire ziekten van de darm en primaire immuundeficiënties. Er kan dan een gepaste behandeling worden ingesteld. Tevens vraagt de groep van *FOXP3*- onafhankelijke AIE om verder onderzoek naar nieuwe invalshoeken voor diagnostiek en behandeling.

6.4 Behandeling van AIE

De meeste vormen van AIE reageren goed op behandeling met corticosteroiden of azathioprine. Symptomatische behandeling van de meest ernstige vorm van AIE, IPEX syndroom, begint met het garanderen van voldoende vocht- en nutriëntinname door middel van elementaire enterale of parenterale voeding. De meest effectieve causale behandeling voor IPEX syndroom is T-celgerichte immuunsuppressie met cyclosporine of tacrolimus.[22,23] Vaak worden deze middelen gecombineerd met azathioprine en/of glucocorticoïden. De beste resultaten tot nu toe werden behaald met tacrolimus.[21,23] Echter, het middel blijkt bij sommige patiënten niet effectief. Bovendien heeft dit middel een aantal ernstige bijwerkingen, waaronder nierbeschadiging. Er zijn aanwijzingen dat tacrolimus onder sommige omstandigheden autoimmuniteit in de hand werkt. In hoofdstuk 4 wordt een aantal transplantatie patiënten beschreven, die onder tacrolimus immunosuppressie een allo-immuun cytopenie ontwikkeld. Aan de hand van de literatuur wordt ingegaan op de mogelijke rol van tacrolimus in het ontstaan van deze hematologische afwijking. In de literatuur komen 3 hypotheses naar voren die een verklaring voor het ontwikkelen van de cytopenie onder tacrolimus immunosuppressie kunnen vormen: Allereerst kan de invloed van het middel op de functie van de thymus een rol spelen. Tevens kan het middel een effect hebben op celdood van geactiveerde T-cellen en tot slot kan het middel naast T-effector cellen ook regulerende T-cellen blokkeren waardoor er een immunologische disbalans ontstaat. Deze hypotheses dienen in experimentele setting verder onderzocht te worden. Recent is ervaring opgedaan met een ander immuunsuppressivum: sirolimus.[24] De eerste klinische resultaten lijken positief. Een moleculaire studie waarbij tacrolimus en sirolimus met elkaar werden vergeleken, ondersteunt het gebruik van sirolimus voor de behandeling van AIE. Het middel onderdrukt de cytokine productie van T-effector cellen in dezelfde mate als tacrolimus. Beide middelen hebben met name een effect op Th-1 cytokine interferon gamma. Naast het effect op de cytokine productie heeft sirolimus een duidelijk remmend effect op de proliferatie van T-effector cellen. Ondanks dat de studie de eerste moleculaire ondersteuning van het gebruik van tacrolimus en sirolimus voor AIE verschaft, is meer kennis over het werkingsmechanisme van deze geneesmiddelen bij AIE van belang, zodat een specifiekere aanpak van AIE mogelijk wordt. Dit is urgent, omdat de resultaten van beenmergtransplantatie nog lang niet optimaal zijn. Hierbij kan gedacht worden aan onderzoek naar het effect van de middelen op de oriëntatie van de immuunrespons of op de inductie van regulerende cellen. Verder kan de biologische activiteit ex-vivo worden

onderzocht in de darmmucosa. Gezien beide immunosuppressoren met name effect hebben op Th-1 cytokine IFN gamma, zou gezocht kunnen worden naar nieuwe middelen die ook Th-2 cytokine productie onderdrukken. Dit is met name van belang voor de IPEX patiënten uit de groep waarbij de enteropathie gecombineerd wordt met immunoallergische reacties. Naast immunosuppressieve therapie wordt er gezocht naar alternatieve behandelmethoden voor AIE. Beenmergtransplantatie is tot op heden de enige mogelijkheid tot genezing. De eerste pogingen met myeloablatieve conditionering hadden slechts matig succes: patiënten overleden in het eerste jaar na transplantatie. Recente benaderingen zonder myeloablatie hebben de uitkomst sterk verbeterd. Deze beenmergtransplantaties zijn minder toxisch en genereren een langdurige stabiele periode; 7 patiënten met een follow-up van maximaal 4 jaar zijn gerapporteerd.[1] Wanneer beenmergtransplantatie vroeg uitgevoerd wordt, kan onomkeerbare beschadiging van het pancreas of de schildklier door de autoantistoffen voorkomen worden.[1] Uitgebreid onderzoek is gaande naar mogelijkheden voor immunotherapie met regulerende T-cellen. Genmodificaties bij muizen hebben ectopische *FOXP3*-expressie mogelijk gemaakt door effector-T-cellen te reprogrammeren.[25] De gegenereerde cellen vertonen veel eigenschappen van de conventionele regulerende T-cellen. Twee studies beschrijven een soortgelijk proces bij menselijke cellen. Tot op heden blijkt *FOXP3*-expressie echter bij menselijke cellen onvoldoende suppressieve capaciteiten aan de regulerende T-cellen te geven.[25]

6.5 Conclusie

Auto-immuun enteropathie is een zeldzame oorzaak voor immunologisch gemedieerde chronische diarree op de kinderleeftijd. Het is een zeer ernstige ziekte waaraan patiënten vaak op jonge leeftijd overlijden. In de afgelopen jaren is meer duidelijk geworden over de pathofysiologie en therapeutische mogelijkheden van AIE. Meer duidelijkheid over de pathofysiologie van AIE is van belang voor een betere/ gerichtere diagnostiek en verbetering van therapeutische mogelijkheden. Daarnaast geeft het belangrijke informatie over de normale werking van de darm. Dit proefschrift heeft hieraan een bijdrage geleverd door dieper in te gaan op de moleculaire achtergrond van AIE. Het toont aan dat regulerende T-cel functie een zeer belangrijke rol speelt in het ontstaan van AIE, echter de resultaten suggereren dat er ook andere factoren een rol kunnen spelen. Bij sommige patiënten ligt hieraan een mutatie in het *FOXP3*-gen ten grondslag, bij anderen is de oorzaak onduidelijk. Verder ondersteunen de data die in het proefschrift worden gepresenteerd de veronderstelling, dat regulerende T-cel functie beïnvloed kan worden door omgevingsfactoren. Dit heeft belangrijke consequenties voor de manier waarop wij denken dat de cellen functioneren en schept daarmee nieuwe mogelijkheden voor therapeutische beïnvloeding. Het proefschrift beschrijft een veel grotere variatie in klinische presentatie van AIE dan tot op heden bekend was. Er kan echter op basis van de gegevens geen duidelijke relatie met het genotype van de patiënten worden gemaakt.

Wel geeft het aan dat de ziekte bij een veel groter aantal patiënten dient te worden overwogen. Tot slot gaat het proefschrift nader in op de huidige immunosuppressieve behandeling van de ziekte, waarbij de eerste moleculaire studie is gedaan die het gebruik van sirolimus voor de aandoening ondersteunt. Toename van kennis op deze gebieden is van groot belang voor de prognose van kinderen met AIE, maar ook zal het leiden tot een beter begrip van de immunologie van de darm.

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CHAPTER 7

APPENDICES

7.1 List of Abbreviations

AIE	AutoImmune Enteropathy
Akt	Ak thymoma
APC	antigen presenting cell
BMT	bone marrow transplantation
Bp	base pair
cAMP	cyclic andenoside monophosphate
CaN	calcineurin
CD	cluster of differentiation.
cDNA	complementary DNA
CD45RA+	naive T-cell
CD45RO+	memory T-cell
CMV	cytomegalo virus
CREB	cAMP response element-binding protein
CsA	cyclosporine A
Dim	Diminished
DNA	Deoxyribonucleic acid
Ebi	epstein barr virus induced
4EBP	4E binding protein
EBV	epstein barr virus

EIF	Eukaryotic Initiation Factor
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence Activated Cell Sorting
FITC	fluorescein isothiocyanate
FK506	tacrolimus
FKBP	FK506 binding protein
FKH	forkhead
FOXP3	forkhead box protein 3
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
GAGE1	G antigen 1
GI	gastro-intestinal
GVHD	graft versus host disease
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHV	human herpes virus
Hi	high
HRP	horseradish peroxidase
HSCT	hematopoietic stem cell transplantation
IBD	inflammatory bowel disease.
IDDM	insulin dependent diabetes mellitus
IFN-γ	interferon-gamma
Ig	immune globulin
IL	interleukine
IL2RA	Interleukine 2 receptor alpha
IPEX	immune dysregulation polyendocrinopathy autoimmune enteropathy X-linked
JAK	janus kinase
JNK	c-Jun N-terminal kinase
KO	knockout
M	mol
(m)Ab	(micro)antibody
MACS	magnetic cell sorting
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
mL	milliliter
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
μL	microliter
NF-AT	nuclear factor of activated T-cells
PBM(N)C	peripheral blood mononuclear cell

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
Pi3K	phosphatidylinositol-3 kinase
PTLD	post-transplant lymphoproliferative disorder
RA	retinoic acid
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	real time
STAT	Signal Transducers and Activators of Transcription
T	thymus
Tc	cytotoxic T-cell
TCR	T-cell receptor
TGF-β	transforming growth factor-beta
Th	T-helper
TLR	toll like receptor
TNF	tumor necrosis factor
Tr1	T-regulatory 1
Tregs	regulatory T-cells
SDS	sodium dodecyl sulfate
SRL	sirolimus
UTR	untranslated region
WT	wild type

7.2 List of Publications

1. Lacaille F, **Moes N**, Hugot JP, Cézard JP, Goulet O, Ruemmele FM. Severe dysimmune cytopenia in children treated with tacrolimus after organ transplantation. Am J Transpl 2006; 6:1072-6.

2. Torgerson TR, Linane A, **Moes N**, Anover S, Matéo V, Rieux-Laucat F, Hermine O, Vijay S, Gambineri E, Cerf-Bensussan N, Fischer A, Ochs HD, Goulet O, Ruemmele FM. A novel mutation in an upstream, non-coding region of the FOXP3 gene leads to a variant of the IPEX syndrome with severe allergy. Gastroenterology 2007;132(5):1705-17.

3. Ruemmele FM, **Moes N**, Patey N Rieux-Laucat F, Goulet O. Clinical and molecular aspects of autoimmune enteropathy and immune dysregulation, polyendocrinopathy autoimmune enteropathy X-linked syndrome. Current Opinion in Gastroenterology 2008;24:742–748.

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8. **Moes N**, Begue B, Garnier-Lengliné H, Canioni D, Rieux-Laucat F, Goulet O, Cerf-Bensussan N, Ruemmele F.M. Rapamycin therapy for severe autoimmune enteropathy - analysis of the molecular mechanisms. Submitted.

9. **Moes N**, Ruemmele FM. Autoimmune enteropathies- new insights. JPGN. In preparation.

7.3 Curriculum Vitae

Nicolette Moes werd op 1 juni 1981 geboren te Leidschendam. Na het behalen van haar middelbare school diploma aan het Praedinius Gymnasium te Groningen (gemiddeld cijfer $>$ 8) begon zij in 1999 met de studie Geneeskunde aan de Rijksuniversiteit van Groningen. Haar propedeutisch examen werd cum laude behaald en ook haar artsexamen werd met een gemiddeld cijfer van een 8 afgerond. Tijdens haar studie heeft zij in het kader van een Erasmus beurs gedurende 3 maanden een klinische stage kindergeneeskunde gevolgd in Caen (Frankrijk) onder supervisie van Professor dr. J.F. Duhamel. Keuzecoschap en wetenschappelijke stage werden uitgevoerd onder begeleiding van Prof. dr. J. Schmitz en Prof. dr. F.M. Ruemmele bij de afdeling kindergastro-enterologie van het Hôpital Necker Enfants Malades te Parijs (Frankrijk). Na het behalen van de artsenbul in november 2005 ging zij in Parijs verder met wetenschappelijk onderzoek in het INSERM laboratorium U793 onder begeleiding van Prof. dr. F.M. Ruemmele en Prof. dr. E.H.H.M. Rings. Dit onderzoek werd mogelijk gemaakt door de Europese Leonardo da Vinci post-graduate grant. Gedurende deze periode heeft zij tevens de Master 'Immunologie Approfondie' gevolgd aan de Universiteit Paris Descartes V in samenwerking met het Instituut Pasteur. Zij behaalde in juni 2007 haar diploma. In datzelfde jaar startte zij met haar promotietraject aan de RUG bij de afdeling kindergastroenterologie van het UMCG in samenwerking met de afdeling kindergastroenterologie van het Hôpital Necker Enfants Malades en het laboratorium INSERM U793 te Parijs (promotores: Prof. dr. E.H.H.M.Rings en Prof. dr. F.M. Ruemmele). De promotie werd gefinancierd door middel van een Zonmw AGIKO fellowship en een Nutricia Research Grant. Nicolette presenteerde de resultaten van haar onderzoek op verschillende internationale bijeenkomsten. In 2007 won zij de John Harries prijs voor beste presentatie in de gastroenterologie op het ESPGHAN (European Society of Paediatric Gastroenterology, Hepatology and Nutrition) congres te Barcelona. Sinds 2011 is zij trainee member van deze organisatie. Voor de praktische uitvoering van het promotiewerk was zij gedurende 1,5 jaar gedetacheerd in Parijs. In oktober 2008 keerde zij terug naar Groningen om te starten met haar klinische opleiding tot kinderarts in het UMCG (opleider: Prof. dr. E. Duiverman opgevolgd door mr. dr. A.E.E. Verhagen). Van oktober 2010 tot april 2012 vervolgde zij haar opleiding in het Martini Ziekenhuis te Groningen (opleider: dr. W.B. Geven). Gedurende haar opleiding was Nicolette actief in het bestuur van de junior afdeling van de Nederlandse vereniging van kindergeneeskunde (NVK) en de de regionale opleidingscommissie. Tevens maakte zij deel uit van het NVK –TULIPS (Training Upcoming Leaders in Paediatric Research) PhD curriculum 2011-2012. Van april 2012 tot april 2013 voltooit zij haar opleiding tot kinderarts in het UMCG. Nicolette hoopt zich nadien verder te specialiseren in de kinder maag-darm leverziekten met als interessegebied immunologische ziekten van de darm. Ze hoopt binnen dit vakgebied patientenzorg met wetenschappelijk onderzoek te kunnen combineren.

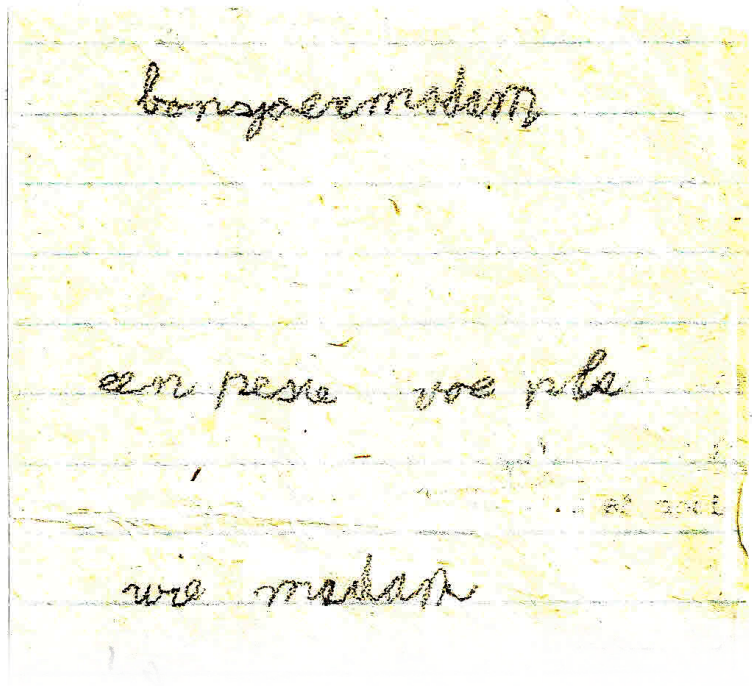

Figure 1. The author's first French writing (8 years old). Order for bread at the grocery store. 'Bonsjoermadam. Een pesie voe ple. Wie madam.' Translation:'Bonjour madame. Un pain s'il vous plait. Oui Madame.

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De patiënten en hun ouders/ Les patients et leurs parents/ The patients and their parents :
Dit proefschrift is opgedragen aan de patiënten met auto-immuun enteropathie en hun ouders.
Cette thèse est dédiée aux patients atteints d'entéropathie autoimmune et à leurs parents.
This thesis is dedicated to patients with autoimmune enteropathy and their parents.

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'Il en faut peu pour être heureux
Vraiment très peu pour être heureux
Chassez de votre esprit
Tous vos soucis
Prenez la vie du bon côté
Riez, sautez, dansez, chantez'

- Baloo, le livre de la jungle, Disney